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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,307,128**, on May 25, 2000, by **QUEEN'S UNIVERSITY AT KINGSTON** and **OREGON HEALTH SCIENCES UNIVERSITY**, assignee of Richard Oko and Peter Sutovsky, for "PT32 Sperm Protein".

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PT32 Sperm Protein, Sperm c-Yes, Oocyte Cytoplasmic c-Yes, and Uses Thereof

Inventors: Richard Oko
Peter Sutovsky

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds, awarded by the National Institutes of Health (Grant No. R-21, RR14293-01) and by the United States Department of Agriculture (New Investigator Award #99-35203-7785). The U.S. Government has certain rights in this invention.

Field of the Invention

The invention relates to proteins of the mammalian sperm and oocyte, and uses thereof, e.g., in enhancing fertility and in contraception.

Background of the Invention

Oocyte activation in mammals encompasses the resumption of second meiosis and the activation of anti-polyspermy defense, which are accompanied by calcium oscillations periodically crossing oocyte cytoplasm (reviewed by Schultz, R. M., and Kopf, G. S., "Molecular basis of mammalian egg activation," in *Current Topics in Developmental Biology*, Pedersen, R. A., and Schatten, G. P., (eds), Vol. 30, Academic Press Inc., San Diego, (1995) pp. 21-62). In bovine and other mammals, the fertilization-induced oocyte activation is also accompanied by the assembly of nuclear pore complexes (NPC) into the cytoplasmic annulate lamellae (AL), and by the insertion of NPCs into a *de novo*-formed nuclear envelope (NE) of the female and male pronuclei (Sutovsky et al., *J. Cell Sci.* 111:2841-2854 (1998)). Three hypotheses were offered to explain the sperm-induced oocyte activation in mammals: The conduit, or calcium bomb hypothesis (Jaffe, L. F., *Ann. N.Y. Acad. Sci. 339*:86-101 (1980)) implicates the direct, sperm-generated "injection" of Ca^{2+} ions into oocyte cytoplasm at

fertilization. The receptor hypothesis (e. g. Jones, K. T., and Whittingham, D. G., *Dev. Biol. 178*:229-237 (1996); Swann, K., *Development 110*:1295-1302 (1990)) maintains that the specific receptors on the sperm and oocyte plasma membranes activate a signaling cascade leading to the release of Ca^{2+} from internal stores in oocyte ER. Finally, the oscillogen hypothesis favors a soluble oscillogenic factor, presumably a polypeptide, which is released from the sperm head into the oocyte cytoplasm at the time of gamete fusion (Kimura, Y., *et al., Biol. Reprod. 58*:1407-1415 (1998); Parrington, J., *et al., Nature 379*:364-368 (1996); Perry, A. C. F., *et al., Biol. Reprod. 60*:747-755 (1999)). Although there is a substantial amount of data in favor of each of the above hypotheses, and each of them may be relevant to certain animal taxa, recent studies seem to support the validity of the oscillogen hypothesis in mammals. The actual mechanism by which the spermatozoon introduces the oscillogenic molecules into oocyte cytoplasm is not known.

Perinuclear theca (PT) is a cytoskeletal coat of the mammalian sperm nucleus that is inserted between the nuclear envelope and the sperm plasma membrane (Bellvé, A. R., *et al., Biol. Reprod. 47*:451-465 (1992); Courtens, J. L., *et al., J. Ultrastruct. Res. 57*:54-64 (1976); Lalli, M., and Clermont, Y., *Am. J. Anat. 160*:419-434 (1981); Oko, R., and Clermont, Y., *Biol. Reprod. 39*:673-687 (1988)). During spermiogenesis, the PT attaches the acrosomal vesicle to the sperm nucleus and may be involved in shaping it (Oko, R., and Maravei, D., *Biol. Reprod. 50*:1000-1014 (1994); Oko, R., and Maravei, D., *Microsc. Res. Tech. 32*:520-532 (1995); Oko, R., and Clermont, Y., "Spermiogenesis," in *Encyclopedia of Reproduction*, Knobil, E. and Neil, J. D., (eds.), Vol. IV, Academic Press Inc., San Diego (1998) pp. 602-609). At fertilization, the PT is removed from the sperm nucleus with the aid of oocyte's cortical microvilli (Sutovsky *et al., Dev. Biol.* 188:75-84 (1997)). Otherwise, an intact PT would constitute an unsurpassable hurdle preventing the access of the zygotic cytoplasm to the sperm nucleus, which at that time undergoes the remodeling into a male pronucleus. Recent studies of infertile men suffering from globozoospermy (Battaglia, D. E., *et al., Fertil. Steril. 68*:118-122 (1997); Edirisinghe, W. R., *et al., Hum. Reprod. 13*:3094-3098 (1998); Rybouchkin, A., *et al.,. Hum. Reprod.*

11:2170-2175 (1996)), a rare spermatogenic disorder in which the absence of PT causes the round shape of the sperm nucleus (Escalier, D., *Int. J. Dev. Biol. 34*:287-297 (1990)), demonstrated that such spermatozoa fail to induce oocyte activation after intracytoplasmic sperm injection (ICSI). Human and non-human primate oocytes are activated by ICSI with normal spermatozoa (Hewitson, L. C., *et al., Biol. Reprod. 55*:271-280 (1996); Palermo, G., *et al., Lancet 340*:17-18 (1992); Sutovsky, P., *et al., Human Reprod.* 14:2301-2312 (1996); Van Steirteghem, A., *et al., Hum. Reprod. 8*:1061-1066 (1993)) and the intracytoplasmic injection of crude (Swann, K., *Development 110*:1295-1302 (1990)) or partially purified (Kimura, Y., *et al., Biol. Reprod. 58*:1407-1415 (1998); Perry, A. C. F., *et al., Biol. Reprod. 60*:747-755 (1999)) sperm extracts activates rodent oocytes.

Summary of the Invention

The present invention is derived, at least in part, from the observation that, even though the PT is removed from the sperm nucleus at the egg surface, it is incorporated completely into oocyte cytoplasm, where it dissolves concomitantly with the progress of pronuclear development. This observation conforms with the increased oocyte activation rates that are obtained after intracytoplasmic injections of pure PT extracts into bovine oocytes, as compared with sham-injected oocytes. The activated oocytes injected with PT-extracts, but not the control, sham-injected, oocytes, displayed the patterns of the nuclear pore complex (NPC) and annulate lamellae (AL) assembly typical of natural fertilization. Furthermore, secondary spermatozoa prevented from entering the oocyte cytoplasm by polyspermy block still show PT release from any part of the sperm head that has fused with the oolemma. Ultrastructural studies showed the dissolution of PT in the oocyte cytoplasm during monospermic fertilization and polyspermy. Taken together, these data support the view that sperm PT harbors the oocyte activating-factor(s) and provide a mechanism for the release of oscillogen(s) from the sperm head into oocyte cytoplasm at fertilization. As discussed below, the sperm perinuclear theca protein PT32 is one such oscillogen,

which interacts with the protein tyrosine kinase c-Yes during spermatogenesis and fertilization.

Accordingly, the invention features an isolated polypeptide that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and (b) at least three domains (e.g., 4, 5, 6, 7, 8, 9, 10, 11, or 12 or more domains), each domain comprising the sequence YGXPPXG (SEQ ID NO:3), wherein Y represents a Tyrosine residue, G represents a Glycine residue, L represents a Leucine residue, A represents an Alanine residue, X represents any amino acid residue, and P represents a Proline residue. Optionally, some or all of the YGXPPXG (SEQ ID NO:3) domains may include additional amino acid residues (e.g., 1, 2, 3, 4, or 5 additional residues) flanking SEQ ID NO: 3. In various embodiments, the polypeptide has a molecular weight of about 32 kDa (e.g., about 28-33kDa); the polypeptide binds to tyrosine kinase c-Yes; and/or the polypeptide induces oocyte activation. Included within the invention are fragments of the polypeptide that are (i) antigenic, (ii) biologically active, and/or (iii) able to bind to the protein tyrosine kinase c-Yes. An "antigenic" fragment is a portion of the polypeptide which is capable of eliciting an immune response in a host and capable of interacting with antibodies or immune cells *in vitro* or *in vivo*. A "biologically active" fragment is a portion of the polypeptide which is capable of inducing oocyte activation alone or by interacting with other polypeptides, such as tyrosine kinase c-Yes or tyrosine kinase c-Yes adaptor proteins. Typically, such a fragment contains at least 3 (typically, at least 10 or all) of the YGXPPXG domains (SEQ ID NO:3), along with a PPPGY domain (SEQ ID NO:1) or LPPAY domain (SEQ ID NO:2).

An exemplary polypeptide of the invention is PT32, which has the sequence of SEQ ID NO:5, illustrated in Fig. 1, or conservative variants thereof.

Peptidomimetics of the aforementioned polypeptides also are included within the invention. In Morgan et al. ("Approaches to the discovery of non-peptide ligands for peptide receptors and peptidases." Annual Reports in Medicinal Chemistry. Ed. F.J. Vinick. San Diego: Academic Press, 1989, pp. 243-252), peptide mimetics are defined as "structures which serve as appropriate substitutes for peptides in interactions with receptors and enzymes. The mimetic

must possess not only affinity, but also efficacy and substrate function." For purposes of this disclosure, the terms "peptidomimetic" and "peptide mimetic" are used interchangeably according to the above-excerpted definition. That is, a peptidomimetic exhibits function(s) of a peptide, without restriction of structure. Peptidomimetics of the present invention, i.e., analogues of biologically active fragments of PT32 or c-Yes, may include amino acid residues or other moieties which provide the functional characteristics described herein.

The invention also features isolated polynucleotides encoding the aforementioned polypeptides. As used herein, "polynucleotide" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA, including cDNA, genomic DNA, and synthetic DNA, or modified RNA or DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. "Polynucleotides" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single-and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms of the polynucleotides typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

Fragments of the polynucleotides of the present invention may be used as hybridization probes for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the polynucleotides or similar function to the encoded polypeptides. Probes of this type preferably

have at least 15 bases, and may contain, for example, 18, 20, 25, 30, or 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promoter regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of cDNA, genomic DNA or mRNA to determine to which members of the library the probe hybridizes.

An exemplary polynucleotide of the invention includes the sequence of SEQ ID NO: 4, illustrated in Fig.1, or degenerate variants thereof. In various embodiments, the invention also includes: (i) a gene that includes such a polynucleotide; (ii) a vector that includes such a polynucleotide or such a gene; and (iii) a host cell that contains such a vector. In addition, the invention includes a method of producing a polypeptide by maintaining the aforementioned host cell under conditions such that the polypeptide is expressed, then collecting the polypeptide.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons). It may further include regulatory elements, such as promoters, enhancers, operators, and repressors, which are useful in promoting, regulating, and/or repressing expression of the gene.

The present invention further relates to variants of the herein described polynucleotides which encode fragments, analogs and derivatives (including semi-synthetic variants) of the polypeptides of the invention. "Variant," as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino

acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be naturally occurring, such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

Also included within the invention are antibodies that specifically bind to the polypeptides of the inventions. Such antibodies can be polyclonal or monoclonal. For example, antibodies that specifically bind to PT32 can be raised by immunizing mammals, e.g., rabbits, with fragments of PT32. For example, antibodies can be raised against a polypeptide having the amino acid sequence TSYRVVFVTSHLVNDPMLSFMMPF (SEQ ID NO:6) or NEALPPAYEAPSAGNT (SEQ ID NO:7). Such antibodies can be used in immunological assays, e.g., Western blotting, ELISAs, and *in situ* immunofluorescent studies.

The aforementioned such antibodies can be formulated with a pharmaceutically acceptable carrier to produce a pharmaceutical composition for use, for example, in immunocontraceptive methods. Other suitable pharmaceutical compositions may include a pharmaceutically acceptable foam and at least one of the following molecules: an antibody that specifically binds to PT32, an antibody that specifically binds to c-Yes, PT32 or a fragment thereof, c-Yes or a fragment thereof, an agonist or antagonist of PT32, and an agonist or antagonist of c-Yes. Such compositions can be used to modulate (enhance or inhibit) oocyte activation in fertility-enhancing or contraceptive methods.

Various methods also are included within the invention. For example, the invention features a method for inducing or enhancing oocyte activation, the

method comprising contacting an oocyte of a mammal (e.g., a human or cow) with at least one of: (1) an isolated polypeptide, such as PT32, that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and (b) at least three domains (e.g., 4, 5, 6, 7, 8, 9, 10, 11, or 12 or more domains), each domain comprising the sequence YGXPPXG (SEQ ID NO:3), or a biologically active fragment thereof, or a peptidomimetic thereof; (2) a c-Yes polypeptide or a biologically active fragment thereof; and (3) globozoospermic sperm, which optionally may be stripped. Oocyte activation can be induced *in vitro* or *in vivo*. In a related method, an oocyte is contacted with a composition consisting essentially of PT32 and/or c-Yes, or a biologically active fragment(s) thereof.

The invention also includes a method for enhancing fertility in a mammal by expressing a biologically active polypeptide of the invention in a germ cell of the mammal (e.g., human, bovine, pig, sheep, goat, monkey, or horse). More particularly, the invention includes a method for treating globozoospermy by expressing in spermatoza an isolated polypeptide that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and (b) at least three domains (e.g., 4, 5, 6, 7, 8, 9, 10, 11, or 12 or more domains), each domain comprising the sequence YGXPPXG (SEQ ID NO:3), Preferably, the polypeptide is a biologically active polypeptide of the invention.

In a related aspect, the invention features a method for enhancing fertility and/or activating an oocyte in a mammal by contacting an oocyte with (e.g., introducing into the oocyte) tyrosine kinase c-Yes, or a biologically active fragment thereof. A biologically active tyrosine kinase c-Yes polypeptide or fragment thereof is capable of phosphorylating a target protein.

Methods for identifying modulators (i.e., enhancers or inhibitors) of oocyte activation also are included within the invention. In an exemplary method, a test compound is contacted with an oocyte, and the oocyte is treated with a biologically active polypeptide of the invention under conditions sufficient to induce oocyte activation in the absence of the test compound. Modulation of oocyte induction then is detected as an indication that the test compound is an modulator of oocyte activation.

A related method for identifying a modulator of oocyte activation includes (A) contacting a test compound with (I) a polypeptide, such as PT32, that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and (b) at least three domains, each domain comprising the sequence YGXPPXG (SEQ ID NO:3), or a tyrosine kinase c-Yes-binding fragment thereof, and (II) tyrosine kinase c-Yes or a PT32-binding fragment thereof, under conditions sufficient to permit in the absence of the test compound binding of the polypeptide or fragment thereof to tyrosine kinase c-Yes or the fragment thereof, and (B) detecting modulation (i.e., enhancement or inhibition) of binding of the polypeptide or the fragment thereof to the tyrosine kinase c-Yes or the fragment thereof as an indication that the test compound is an modulator of oocyte activation.

The invention also provides methods for modulating (i.e, enhancing or inhibiting) fertilization. For example, the invention includes a method for inhibiting fertilization of a mammalian oocyte by inhibiting the interaction of PT32 with tyrosine kinase c-Yes in the oocyte. Such inhibition can include contacting the oocyte with at least one of: (a) an antibody that specifically binds to PT32 and (b) an antibody that specifically binds to tyrosine kinase c-Yes.

In an exemplary immunocontraceptive method, a polypeptide that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and (b) at least three domains, each domain comprising the sequence YGXPPXG (SEQ ID NO:3), or an antigenic fragment of such a polypeptide, is introduced into a mammal (typically a male), such that an immune response is elicited. For example, PT32 can be used in such a method. The polypeptide, or antigenic fragment thereof, elicits an immune response in the mammal, and the biological activity of PT32 endogenous to the mammal is inhibited, thereby inhibiting fertilization.

Optionally, the polypeptide or antigenic fragment thereof is produced as a fusion protein that includes the polypeptide (or antigenic fragment) covalently linked to a second polypeptide. The second polypeptide can be a conventional carrier protein, which preferably is foreign to the host (e.g., ovalbumin or keyhole limpet hemocyanin (KLH)) to facilitate the elicitation of an immune response.

Such fusion polypeptides (or, alternatively, antigenic, non-fusion polypeptides of the invention) can be formulated with a conventional adjuvant to produce a vaccine for administration to a mammal (e.g., human or bovine) in a immunocontraceptive method. Alternatively, the second polypeptide of a fusion polypeptide can be a marker sequence that facilitates detection or purification of the polypeptide of the invention. For example, the marker sequence can be a hexahistidine tag, e.g., supplied by a pQE-9 vector, to provide for purification of a recombinant polypeptide from a prokaryotic (bacterial) host cell. Alternatively, the marker sequence can be a hemagglutinin (HA) tag (i.e., an epitope of an influenza hemagglutinin protein) to facilitate purification from a eukaryotic, e.g., mammalian, host cell (e.g., COS-7) cells. In another example, a green fluorescent protein (GFP) is fused to a polypeptide of the invention to facilitate protein detection using fluorescent methods. A variety of other art-known marker polypeptides can be fused to the polypeptides of the invention to produce fusion proteins.

As an alternative to introducing a polypeptide vaccine into a mammal, a DNA vaccine can be used to elicit an immune response in the mammal. For example, a polynucleotide encoding a polypeptide (e.g., PT32) that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and (b) at least three domains, each domain comprising the sequence YGXPPXG (SEQ ID NO:3), can be administered to the mammal under conditions that permit expression of the polypeptide in the mammal, thereby eliciting an immune response against the polypeptide. Preferably, the polypeptide is not biologically active. Optionally, a DNA vaccine can include a polynucleotide encoding a carrier protein fused to the polypeptide.

The invention also includes several diagnostic methods. For example, the invention includes a method for diagnosing diminished fertility in a mammal by measuring in a germ cell (spermatoza or oocyte) of the mammal the level of (A) tyrosine kinase c-Yes and/or (B) a polypeptide (e.g., PT32) that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO:2) and (b) at least three domains, each domain comprising the sequence YGXPPXG (SEQ ID NO:3). A diminution in the levels of c-Yes

and/or the polypeptide in the germ cell (e.g., by 10%, 25%, 50% or even more) indicates that the mammal suffers from (or is at risk for) diminished fertility.

In a related aspect, the invention provides a method for diagnosing abnormal spermiogenesis in a mammal. The method includes comparing (1) the pattern of the distribution of a polypeptide that includes (a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and (b) at least three domains, each domain comprising the sequence YGXPPXG (SEQ ID NO:3) (e.g., PT32), throughout mature spermatoza of the mammal with (2) the pattern of the distribution of the polypeptide throughout healthy, mature spermatoza, wherein an abnormal distribution pattern is an indication that spermiogenesis in the mammal is abnormal. For example, the failure of PT32 to be localized (i) between the acrosome and the nucleus of the spermatoza and/or (ii) on the post-acrosomal portion of the head of the spermatoza is an indication that spermiogenesis is abnormal in the mammal.

A related method for diagnosing spermiogenesis in a mammal involves comparing (i) the pattern of the distribution of tyrosine kinase c-Yes throughout mature spermatoza of the mammal with (ii) the pattern of the distribution of tyrosine kinase c-Yes throughout healthy, mature spermatoza, wherein an abnormal distribution pattern is an indication that spermiogenesis in the mammal is abnormal.

The invention also features a transgenic non-human mammal whose germ cells contain a disruption in the endogenous gene encoding PT32, *e.g.*, by insertion of a selectable marker sequence at the *PT32* locus, and the disruption results in the lack of expression or function of PT32. Preferably, the non-human mammal is murine or a monkey. Optionally, the animal may be bovine. Such an animal can be used as an animal model for studying human fertility and reproductive biology, as sperm produced from such animals can be expected to be defective (e.g., globozoospermic), and such animals can be expected to have diminished fertility.

Additionally, such an animal can be used as an animal model for screening compounds to identify modulators of oocyte activation. For example, such an method can include contacting an oocyte with (i) sperm produced by the

transgenic non-human mammal, and (ii) a test compound, and detecting an inhibition or enhancement of oocyte activation as an indicator that the test compound is a modulator of oocyte activation. If desired, the transgenic non-human animals of the invention can be used as negative control animals for comparison to wild-type animals.

Also included within the invention is a transgenic non-human mammal whose germ cells contain a disruption in the endogenous gene encoding tyrosine kinase c-Yes, wherein the disruption comprises the insertion of a selectable marker sequence, and wherein the disruption results in the lack of expression or function of the tyrosine kinase c-Yes. Such animals (e.g., mice, monkeys, etc) can be used as animal models in studies of human fertility and reproductive biology.

In a related aspect, the invention includes a method for identifying a modulator of oocyte activation, the method comprising contacting an oocyte of the above-described transgenic non-human mammal having a disrupted c-Yes gene with (i) a test compound and (ii) spermatoza, and detecting inhibition or enhancement of oocyte activation as an indicator that the test compound is a modulator of oocyte activation.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

Brief Description of the Figures

Fig. 1 is a listing of the amino acid and nucleic acid sequences of an exemplary PT32 polypeptide of the invention.

Fig. 2(A) Diagrammatic representation of a mid-sagittal section through the head of a bull spermatozoon showing the three parts of the Perinuclear Theca, i.e., the subacrosomal layer, the postacrosomal sheath and outer periacrosomal layer (OPL). Fig. 2B illustrates the polypeptide SDS-PAGE profile of Perinuclear Theca (PT) extract. All of the major proteins shown have been immunolocalized to the PT and the identity of most of the proteins shown is known.

Fig. 3. Incorporation and dissolution of the sperm perinuclear theca in the cytoplasm of bovine oocytes at fertilization. Bovine oocytes were fertilized with the MitoTracker Green FM-tagged spermatozoa (white; sperm taff mitochondria) and processed for indirect immunofluorescence with a PT-specific antibody pAb 427 (red), the nuclear pore complex-specific antibody mAb 414 (green), and the DNA stain DAPI (blue). (A) An unfertilized, metaphase-H-arrested oocyte shows no labeling with either antibody. (B-D) Removal of the sperm PT (arrows) from the surface of the sperm nucleus during its incorporation into the oocyte cytoplasm. Concomitantly with this process, the female chromosomes (*f*) complete the second meiosis. (E) An early stage male pronucleus with a continuous ring of nuclear pores marking the presence of a newly-reconstituted nuclear envelope, and a clump of PT-derived material in the adjacent cytoplasm (arrow). Note a small stretch of PT (arrowhead) still attached to the male pronucleus, and the nascent female pronucleus (*f*) that is still devoid of nuclear pores. (F) Reconstitution of the nuclear envelope, complete with nuclear pore complexes, on the surface of a developing male pronucleus. This pronucleus overlaps with a clump of PT-derived material in the cytoplasm (arrow). Female chromatin having completed second meiosis marked by the presence of the second polar body (*f*, bottom) and a small nascent female pronucleus (*f*, top). (G-H) Dissolution of the PT (arrows) in the cytoplasm of fertilized bovine oocytes. Similar to their male counterparts, the female pronuclei (*f*) acquire the nuclear envelope and nuclear pore complexes at this stage of pronuclear development. The PT is not detectable in the zygotes containing large, non-apposed (J-K) or apposed (LM) male (*m*) and female (*f*) pronuclei. Note the abundance of the NPC containing annulate lamellae (green) in the cytoplasm of these zygotes. (N) A spontaneous parthenogenote displaying aberrant assembly of NPCs and AL on its nucleus and in its cytoplasm, respectively. Scale bar = 10 μm.

Fig. 4. Intracytoplasmic injection of a single intact spermatozoon (A-F), sperm PT-extracts (G-J), and the calcium-free culture medium (K-M) into bovine oocytes further cultured for 20 hours and processed for indirect immunofluorescence with the anti-PT antibody pAb 427 (red), the NPC-specific antibody mAb 414 (green) and the DNA stain DAPI (blue). (A-C) An intact PT

(arrows) is seen on the surface of the injected sperm nuclei in the non-activated oocytes containing metaphase-II plate of maternal chromosomes (f). (D) Signs of the female chromosome (*f*) decondensation coincided with the dispersal of sperm PT (arrows) in this oocyte. (E) Progression of oocyte meiosis in an oocyte containing a decondensed sperm nucleus with the remnants of PT (arrows) on its surface. (F) A large female pronucleus (*f*) with regular ring of nuclear pores in an oocyte containing single spermatozoon with an intact equatorial segment of PT (arrow), and a completely dissolved subacrosomal PT. (G, H) Oocytes activated by the injection of isolated PT-extracts contain one (G) or two (H) parthenogenetic female pronuclei with a ring of NPCs and the abundant cytoplasmic annulate lamellae (green dots in cytoplasm). (I) An oocyte that failed to activate after the PT-extract injection displays no NPCs or annulate lamellae. (J) An oocyte activated by the injection of boiled PT-extract. (K) A sham-injected inactivated oocyte. (L, M) Sham-injected oocytes, that developed female pronuclei with NPCS, lack the cytoplasmic annulate lamellae typical for the oocytes fertilized by a spermatozoon or activated by the PT-extract. Large cytoplasmic sheets labeled with mAb 414 are often seen in the cytoplasm of such oocytes (M). Scale bar = 10 μm.

Fig. 5. Composition of bull PT extracts used in microinjection experiments. A: SDS-PAGE of bull sperm PT extract showing major PT polypeptides stained with Coomassie blue (lane 2) and compared to molecular mass standards (lane 1). Note the predominant bands of 15-, 25-, 28-, 32-, and 60- kDa. B: Preparative western blot strips of electrophoresis-separated PT extract reacted with three immunization boosts of PT-specific antibody pAb 427, Immune serum from the first boost (lane 3) stains predominantly PT 15 and PT 28, second boost (lane 4) displays high affinity to PT 32 and third boost (lane 5) stains strongly, amongst other bands, PT 36. Lane I shows the molecular mass standards and lane 2 Coomassie blue-staining of the transferred PT extracts.

Fig. 6. Ultrastructural aspects of sperm PT-oocyte interactions during in vitro fertilization in bovine. A: Sperm oolemma binding demonstrated on a cross-section of the acrosomal part of an acrosome-reacted bull sperm head. Several oocyte microvilli (arrows) are bound to the subacrosomal layer of PT

(arrowheads). Continuous inner acrosomal plasma membrane is not discernible in this micrograph. B: Detail of a sperm head shown in Fig. A. The lateral edge of the sperm nucleus appears to be engulfed by two oocyte microvilli (arrows), thus creating a closed space in which the subacrosomal PT (full arrowheads) may mingle with oocyte cytoplasm. Note the remnants of inner acrosomal membrane (empty arrowheads) on the lateral face of this sperm head. C: Removal of the sperm PT during sperm incorporation into oocyte cytoplasm. Note the distinct layers of PT (arrows) peeling off the equatorial segment of this sperm nucleus, and the oocyte microvilli (arrowheads) bound to the innermost PT layer. The outer PT layers have a fuzzy appearance, likely reflecting their progressive dissolution in the cytoplasm. D: Sperm incorporation block caused by the oocyte's anti-polyspermy defense. The acrosomal region and the equatorial segment of this spermatozoon are engulfed by oocyte cytoplasm, whereas a part of the postacrosomal sheath and the axoneme emanate into the perivitelline space. Note the intact PT (arrows) on the non-incorporated part of the nucleus, which contrasts with the absence of PT, decondensation of the sperm chromatin, and formation of the new nuclear envelope (arrowheads) around the incorporated part of the nucleus. E: Detail of Fig. D., showing the persistence of PT on the unincorporated portion of the post-acrosomal sheath (bars). F: Detail of Fig. D, showing the new nuclear envelope (arrows) and the decondensing sperm chromatin in the incorporated portion of this sperm nucleus. (G-I) Binding of the oocyte microvilli (arrows) to the perinuclear theca (arrowheads) of the lysolecithin-demembranated spermatozoa. Note the unusually high number of oocyte microvilli bound to these sperm heads, as compared to the spermatozoa with intact plasma membrane in Figs. A, B. Insert (H) shows multiple demembranated spermatozoa bound to a single, zona-free oocyte, as visualized by DAPI labeling. Scale bars: A=500 nm, B=200 nm.

Fig. 7. Schematic interpretation of the release of PT-anchored oscillogens into oocyte cytoplasm at fertilization. A: Acrosome reaction causes that the inner acrosomal membrane, equatorial segment and the postacrosomal sheath of the sperm head become accessible to the oocyte microvilli once the spermatozoon reaches the perivitelline space. B: Oocyte microvilli fuse with the sperm plasma

membrane at equatorial segment and in the subacrosomal part of PT, effectively exposing the perinuclear theca to oocyte cytoplasm. C: During normal fertilization, the oocyte microvilli drag the perinuclear theca and sperm nucleus into the cytoplasm, and the PT starts to detach from the nuclear surface and slowly dissolves in the cytoplasm (see also Fig. 3C). Cytoplasmic factors such as glutathione (Perreault, S. D., *et al., Dev. Biol. 101*:160-167 (1984); Sutovsky, P., and Schatten, G., *Biol. Reprod. 56*:1503-1512 (1997)) may facilitate the dissolution and dispersion of sperm PT. D: Sperm-incorporation arrest induced by polyspermy block or by microfilament disruption nevertheless results in oocyte activation, as the oocyte microvilli retain their ability to fuse with the sperm plasma membrane, effectively exposing the sperm PT to oocyte cytoplasm (see also Fig. 3D-F). PT from those parts of the sperm head engulfed by the oocyte is then released into oocyte cytoplasm, thus explaining the ability of bull spermatozoa to activate the oocytes in the absence of complete sperm incorporation after cytochalasin B-treatment (Sutovsky, P., *et al., Biol. Reprod. 55*:1195-1205 (1996b)). ES=equatorial segment, IAM=inner acrosomal membrane, NE=nuclear envelope, OAM=outer acrosomal membrane, PM=plasma membrane, PS=postacrosomal sheath, SL=subacrosomal layer of PT. Adapted with publisher's permission from Yanagimachi, 1994, after Bedford and Cooper, 1978.

Figs. 8 A and B illustrate the effects of injection of PT32, perinuclear theca extracts, and bovine serum albumin into oocytes.

Figs. 9 A-H illustrate that PT32 and perinuclear theca extracts induce clustering of c-Yes in oocytes.

Figs. 10 A-F illustrate the pattern of expression of c-Yes and PT32 in sperm and spermatids of bull.

Fig. 11 is a listing of the amino acid sequence of an exemplary c-Yes protein. The sequence of this protein also can be found in the SWISSPROT database under accession number P09324.

Detailed Description of the Preferred Embodiments

"Identity" is a measure of the identity of nucleotide sequences or amino acid sequences. In general, the sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques. *See, e.g.*: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, 1988; BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, 1993; COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, 1987; and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereaux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H., and Lipton, D., SIAM J Applied Math (1988) 48:1073). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipton, D., SIAM J Applied Math (1988) 48:1073. Methods to determine identity and similarity are provided by computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCS program package (Devereux, J., *et al.*, *Nucleic Acids Research* (1984) 12(1):387), BLASTP, BLASTN, FASTA (Altschul, S.F. *et al.*, *J. Molec. Biol.* (1990) 215:403).

As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence, is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of

the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

Similarly, by a polypeptide having an amino acid sequence having at least, for example, 95% "identity" to a reference amino acid sequence, is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxyl terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

The present invention further relates to polynucleotides which hybridize to PT32 as set forth in SEQ ID NO:4 if there is at least 75%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to PT32 as set forth in SEQ ID NO:4. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. Exemplary stringent conditions include hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. The polynucleotides which hybridize to the polynucleotides described herein (e.g., to SEQ ID NO:4), in a preferred embodiment, encode

polypeptides which retain substantially the same function as the PT32 polypeptides of the invention (e.g., binding to c-Yes and/or activation of oocyte induction).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a PT32 polynucleotide under stringent conditions, as set forth in SEQ ID NO:4, and which has an identity thereto, as herein described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotides described herein, for example, for recovery of the polynucleotide or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least 75% identity, *e.g.*, at least 90% identity, and preferably at least a 95% identity, to a polynucleotide which encodes PT32, e.g., the polypeptide of SEQ ID NO: 5 (which can be encoded by the polynucleotide of SEQ ID NO:4), as well as fragments thereof, which fragments have at least 20 bases, and preferably at least 30 or 50 bases, and to polypeptides encoded by such polynucleotides.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention, and the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

Host cells are genetically engineered (e.g., transduced, transformed, or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants, or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences, *e.g.*, derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art, such as Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)(the disclosure of which is hereby incorporated by reference).

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: an LTR or SV40 promoter, the *E. coli lac* or *trp* promoters, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence as described herein, as well as an appropriate promoter or control sequence(s), may be employed to transform an appropriate host to permit the host to express the protein. Introduction of polynucleotides into host cells can be effected by methods

described in many standard laboratory manuals, such as Davis *et al.*, BASIC METHODS IN MOLECULAR BIOLOGY (1986) and Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, or infection.

Examples of appropriate hosts include, without limitation, bacterial cells, such as *Streptococci*, *Staphylococci*, *E. coli*, *Streptomyces* and *Bacillus subtilis*; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 or Sf21 cells; and animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells. Animal cells, particularly human or bovine cells, are preferred. Such host cells can be somatic cells or germ cells; artisans of ordinary skill can readily select a cell type suitable to artisan's purpose.

A variety of expression systems can be used to produce the polypeptides of the invention. Such systems include, *inter alia*, chromosomal, episomal and virus-derived systems, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL (*supra*).

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above.

The constructs include a vector, such as a plasmid or viral vector, into which a polynucleotide sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct also includes regulatory sequences, e.g., a promoter operably linked to the polynucleotide sequence of the invention. Suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pBluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia).

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Exemplary bacterial promoters include *lacI, lacZ*, T3, T7, gpt, lambda P_R, P_L and trp. Exemplary eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retroviruses, and mouse metallothionein-I promoters. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the desired polypeptide. These signal sequences may be heterologous to the polypeptides of the invention.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell (e.g., a germ cell or a somatic cell), or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected any of a variety of art-known methods, such as microinjection, calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to

produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

As used herein, the term "polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. *See*, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T.

E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and non-protein cofactors," *Meth Enzymol* (1990) 182:626-646 and Rattan *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci* (1992) 663:48-62.

The terms "fragment," "derivative," and "analog" when referring to the polypeptides of the invention mean a polypeptide which either retains substantially the same function as a reference polypeptide, *e.g.*, retains the ability to bind to c-Yes or PT32, or which retains a biological activity of the reference polypeptide, *e.g.*, retains the ability to induce oocyte activation.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide, a synthetic polypeptide, or a semi-synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of the invention may be, without limitation, (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which one or more of the amino acid residues includes a substituent group, (iii) one in which the polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), (iv) one in which additional amino acids are fused to the polypeptide, e.g., employed for purification of the polypeptide, (v) one which contains a proprotein sequence, or (vi) one in which a signal sequence is fused to the polypeptide. Such fragments, derivatives, and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides of the present invention include the sequence set forth herein as SEQ ID NO: 5, as illustrated in Fig.1, as well as polypeptides that have at least 75% similarity (preferably at least 75% identity), preferably at least 90% similarity (more preferably at least 90% identity), to such polypeptides, and still

more preferably at least 95% similarity (still more preferably at least 95% identity) to such polypeptides. As known in the art, "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Also included are portions of such polypeptides, including antigenic portions of the polypeptide, which generally contain at least 8 amino acids and more preferably at least 10 amino acids. Biologically active portions of such polypeptides are included, which generally contain at least 5 domains (preferably 6, 7, 8, 9, 10, 11, 12, or more domains) that include the amino acid sequence YGXPPXG (SEQ ID NO:3). Preferably, such portions also contain the amino acid sequence PPPGY (SEQ ID NO:1). Such portions of the polypeptide also are expected to be c-Yes binding portions. If desired, the biological activity of a given polypeptide can be measured by measuring the ability of the polypeptide to activate oocyte induction, e.g., using method described herein. The ability of a particular polypeptide to bind to c-Yes can be measured, if desired, in a conventional assay of protein-protein interactions, e.g., in a co-immunoprecipitation assay, in a two-hybrid assay, or in an *in situ* immunoassay (e.g., as described herein).

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Similarly, fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention. Preferred fragments of the polypeptides of the present invention or fragments of the nucleotide sequence coding therefor, include, for example, truncation polypeptides having the amino acid sequence of a PT32 polypeptide, except for deletion of a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus, or deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred are fragments characterized by structural or functional attributes, such as fragments

that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Other preferred fragments are antigenic fragments, biologically active fragments (including those with a similar activity, or an improved activity, or with a decreased undesirable activity), fragments that are immunogenic in an animal (especially in a human or bull), and fragments that bind to tyrosine kinase c-Yes. A given fragment may have more than one of the aforementioned properties. For example, a given fragment may be both biologically active and antigenic.

The polypeptides of the invention can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989).

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* or the *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous

structural sequence is assembled in an appropriate phase with translation initiation and termination sequences, and, preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein, such as a protein that includes an identification peptide (e.g., a hexahistidine tag) imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotech, Madison, Wis., USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical

disruption, or use of cell lysing agents, such methods are well know to those skilled in the art. If desired, the polypeptides of the invention may be solubilized from plasma membranes in digitonin using conventional techniques.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell 23*:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa, HEK and BHK cell lines. Mammalian expression vectors may comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

The polypeptides of the invention can be recovered and purified from recombinant cell cultures by methods such as those including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, metal affinity chromatography (e.g., Ni-NTA), and lectin chromatography. Optionally, high performance liquid chromatography (HPLC) can be employed in the purification steps. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide.

The present invention also relates to the use of the polynucleotides described herein (e.g., a polynucleotide encoding PT32) for use as diagnostic reagents. For example, detection of a mutated form of PT32 provides a diagnostic tool that can add to, or define, a diagnosis of a disease or susceptibility to a disease which results from under-expression, over-expression or altered expression of PT32. For example, mutations in the PT32 gene can result in alterations in the shape or spermatoza and/or cause (or contribute to) diminished fertility in a mammal. Mutations in the PT32 gene may be detected at the DNA level by a variety of conventional techniques.

Nucleic acids for diagnosis may be obtained from a subject's cells or bodily fluids, such as from spermatoza, blood, urine, saliva, tissue biopsy, or autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR or other amplification techniques prior to analysis. RNA or cDNA may also be used in similar fashion. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to labeled PT32 nucleotide sequences or fragments thereof. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence differences may also be detected by alterations in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing. *See, e.g.*, Myers *et al.*, *Science* (1985) 230:1242. Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method. *See* Cotton *et al.*, *Proc Natl Acad Sci USA* (1985) 85: 4397-4401. In another embodiment, an array of oligonucleotide probes comprising PT32 nucleotide sequences or fragments thereof can be constructed to conduct efficient screening of, *e.g.*, genetic mutations. Array technology methods are well known and have general applicability and can be used to address a variety of questions in molecular genetics including gene expression, genetic linkage, and genetic variability. (*See* for example M. Chee *et al.*, *Science 274*:610-613 (1996)).

The invention also provides a method for assessing the quality of spermatoza. The method involves measuring the level of PT32 expression in the sperm of a mammal (e.g., a human or cattle), and comparing the level of PT32 expression with the level of PT32 expression found in normal mammals of the same species. A depreciation in the level of PT32 expression in the mammal (e.g., a 15%, 25% 50% depreciation or more) indicates that the mammal's sperm are of diminished quality. Mammals having diminished levels of PT32 expression are impaired in their ability to induce oocyte activation. Thus, the invention provides a method for determining whether a mammal has an impaired

ability to induce oocyte activation. Such impairment may result in infertility of the mammal.

Biologically active polypeptides of the invention (e.g., PT32 and/ore c-Yes) can be used in various assisted reproductive techniques (ART), including, but not limited to, intracytoplasmic sperm injection (ICSI) of impaired sperm (e.g., globozoospermic sperm) or of immature sperm; round spermatid injection (ROSI); and nuclear transfer (i.e., cloning) methods using somatic, embryonic, or germ cells. Such methods are well known in the art, as described, for examples in U.S. Patent Nos. 6,050,935; 5,935,800; 5,908,380; 5,897,988; 5,882,928; 5,770,363; 5,691,194; 5,627,066; 6,066,725; 6,013,857; 6,011,197; 5,994,619; 5,952,222; 5,945,577; 5,942,435; 5,907,080; 5,863,528; 5,858,963; 5,849,991; 5,843,754; 5,817,453; and 5,741,957, and Eyestone and Campbell, *J. Reprod. Fert. Supp.* 54:489-497 (1999), each of which is incorporated herein by reference. *See also* Shiga *et al. Theriogenology* 52(3):527-35 (1999); Nour *et al., Theriogenology* Feb;51(3):661-6 (1999); Booth *et al. Theriogenology* 51(5):999-1006 (1999); Trounson *et al. Reprod Fertil Dev.* 10(7-8):645-50 (1998); Karnikova *et al. Reprod Nutr Dev.* 38(6):665-70 (1998); Wolf *et al. Biol Reprod.* Feb;60(2):199-204 (1999); Wolf *et al., J Biotechnol.*65(2-3):99-110 (1998); Peura *et al., Mol Reprod Dev.* 50(2):185-91 (1998); Zakhartchenko et al. Mol Reprod Dev. 1997 Nov;48(3):332-8.; Wells et al., Biol Reprod. 1997 Aug;57(2):385-93; Taniguchi *et al.* J Vet Med Sci. 1996 Jul;58(7):635-40; Ouhibi et al. Reprod Nutr Dev. 1996;36(6):661-6; Prochazka and Fiser Reprod Nutr Dev. 1995;35(6):695-701;Yang et al. Mol Reprod Dev. 1993 May;35(1):29-36; First and Prather, J Reprod Fertil Suppl. 1991;43:245-54; and Czolowska et al. J Cell Sci. 1986 Aug;84:129-38, each of which is incorporated herein by reference..

The use of recombinant proteins, such as recombinant PT32 and/or recombinant c-Yes, in ART offers advantages not provided by conventional methods for artificial activation of oocytes. Oocyte activation with crude sperm extracts may introduce into the oocyte sperm components that normally are removed before the sperm enters the egg, and which may be detrimental to embryonic development (e.g., the acrosome). In addition, sperm can carry

viruses such as HIV and SIV, which may be propagated by using crude sperm extracts. Furthermore, ethical concerns are raised by the use of sperm extracts from male donors (i.e., "cytoplasmic fathers") in carrying out ICSI or ROSI. Such concerns can be avoided by using a recombinant protein, e.g., PT32 and/or c-Yes, to activate an oocyte.

The PT32 and c-Yes proteins also are useful in the field of contraception. Specifically, the PT32 and/or c-Yes protein can be used as targets in conventional immunocontraception methods. A variety of such methods have been described, and can readily be modified for use with the PT32 and c-Yes polypeptides described herein. Examples of conventional immunocontraceptive methods are disclosed in U.S. Patent Nos. 6,045,799; 6,027,737; 6,013,770; 5,989,550; 5,989,549; 5,916,768; 5,753,231; and 5,672,488, each of which is incorporated herein by reference. Generally, an immunocontraceptively effective dose of the PT32 or c-Yes protein (or an antigenic fragment thereof) is administered to the mammal (e.g., human) to be treated. Preferably, a chimeric protein containing all or an antigenic portion of PT32 or c-Yes is administered to the mammal in a contraceptively effective dosage. The chimeric protein includes a carrier protein or fragment thereof, such as ovalbumin or KLH. The protein(s) of the present invention may be administered with a suitable diluent, adjuvant, carrier or in a depot (slow release) formulation to allow prolonged exposure of the protein to the host mammal's immune system. A contraceptively effective dosage is a dosage sufficient to elicit the production of an immune response (e.g., antibody or immune cell production) in the mammal.

The polypeptides of the invention, e.g., PT32, can also be used to identify test compounds (e.g., agonists and antagonists, such as small molecules and polypeptides) that bind to the polypeptides of the invention, or to measure the ability of test compounds to bind to the polypeptides. Such assays can be carried out, for example, in cells or in cell-free preparations. The test compound can be a natural, synthetic, or semi-synthetic substance, e.g., a structural or functional mimetic. *See* Coligan *et al., Current Protocols in Immunology* 1(2): Chapter 5 (1991). In a preferred embodiment, the invention includes a method for identifying test compounds that are agonists or antagonists (i.e., that promote or

inhibit) the binding of PT32 to c-Yes. Such test compounds can be identified with conventional methods. For example, conventional two-hybrid methods for identifying compounds that affect protein-protein interactions are well known in the art and can be used in the invention, as described, for example, in U.S. Patent Nos. 5,965,368; 5,955,280; and 6,004,746, each of which is incorporated herein by reference.

An exemplary potential antagonist is an antisense construct prepared through the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes the polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix - *see* Lee *et al.*, *Nucl. Acids Res. 6*:3073 (1979); Cooney *et al.*, *Science 241*:456 (1988); and Dervan *et al.*, *Science 251*:1360 (1991)), thereby preventing transcription and the production of the targeted polypeptide (e.g., PT32). The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the polypeptide (antisense --Okano, J. *Neurochem. 56*:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of a polypeptide of the invention.

The polypeptides, antibodies, or test compounds (e.g., antagonists or agonists) of the invention may be employed in combination with a suitable pharmaceutical carrier or device. Such compositions comprise a therapeutically effective amount of the polypeptide or test compound, and a pharmaceutically acceptable carrier or excipient. Examples of such carriers and excipients include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. Optionally, the polypeptide, antibodies, or test compounds can be formulated with a carrier and/or device conventionally used

for delivering contraceptive or fertility-enhancing agents. For example, conventional foams, gels, sponges, suppositories, creams, tablets, controlled delivery devices, vaginal-soluble waffles, ointments, lotions, sprays, jellies, patches, and lubricants (e.g., for condoms, diaphragms, cervical caps), and the like can be used in conjunction with the molecules of the invention. Suitable carriers and devices that can be modified to contain the molecules of the invention are well known in the art. Without limitation, examples are described in U.S. Patent Nos. 5,725,870; 5,527,534; 4,795,761; 6,063,395; and 6,056,966, all of which are incorporated by reference herein. Such foams, creams, and the like can be administered, e.g., intravaginally, to a mammal to provide a contraceptive (e.g., a contraceptive barrier) in a contraceptive method (e.g., to inhibit fertilization), or to provide a fertility-enhancing agent in a method for enhancing fertility. As desired, the formulation can be optimized to suit the mode of administration. Polypeptides and other molecules of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic or contraceptive compounds.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the compounds of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the various pharmaceutical agents described herein will be administered in an amount of at least about 10 μg/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 μg/kg

to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The polypeptides of the invention, and antagonists or agonists that are polypeptides, may be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy." For an overview of gene therapy, *see* Chapter 20, "Gene Therapy and Other Molecular Genetic-based Therapeutic Approaches," (and references cited therein) in *Human Molecular Genetics*, T. Strachan and A P Read, BIOS Scientific Publishers Ltd (1996).

Thus, for example, cells (particularly spermatoza) may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a mammal (e.g., a human or a bovine mammal) for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviruses, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, *et al.*, *Biotechniques 7(9)*:980-990 (1989), or any other promoter (*e.g.*, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the

histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters (e.g., an adenoviral major late promoter), thymidine kinase (TK) promoters (e.g., a Herpes Simplex Virus thymidine kinase promoter), B19 parvovirus promoters, a respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; and human growth hormone promoters. The promoter also may be the native promoter which controls the genes encoding the polypeptides. Preferably, the promoter is a testis- or sperm-specific promoter to facilitate selective expression of the polypeptide in germ cells of the mammal. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The retroviral vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells that may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy*, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral particles that include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral particles then may be employed to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells. Preferably, the cell is a testicular cell, to facilitate expression of the polypeptide in germ cells of the mammal.

The present invention also provides a method for identifying polypeptides related to the polypeptides (e.g., PT32) of the present invention. These related polypeptides may be identified by homology to a polypeptide of the present invention, by low stringency cross hybridization, or by identifying polypeptides that interact with related natural or synthetic binding partners (e.g., c-Yes) and/or elicit physiological effects as the polypeptides of the present invention (e.g., induction of oocyte activation). The detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (*e.g.*, Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to, and can hybridize with, a particular location on an individual mammalian chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA encoding polypeptide of the invention (e.g., PT32). Computer analysis of the cDNA typically is used to rapidly select primers that do not span more than one exon in the genomic DNA. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, *see* Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man* (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (co-inheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected subjects. If a mutation is observed in some or all of the affected subjects, but not in any normal subjects, the mutation is likely to be the causative agent of the disorder (e.g., infertility or abnormal spermiogenesis).

The polypeptides of the invention, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by introducing the polypeptides into an animal, e.g., a non-human mammal, such as a rabbit or mouse. The antibody so obtained will then bind the polypeptides of the invention. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides, as described further herein. For preparation of monoclonal antibodies, any technique that provides antibodies produced by continuous cell line cultures can be used.

Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature 256*:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, 1983, *Immunology Today 4*:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, *et al.*, 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (*e.g.*, U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptides of the invention. Also, transgenic animals (*e.g.*, mice) may be used to express humanized antibodies to immunogenic polypeptides of the invention.

The above-described antibodies may be employed, for example, to isolate or to identify cells expressing the polypeptide, or to purify the polypeptide by affinity chromatography. Alternatively, such antibodies can be used in immunoassays (e.g., *in situ* immunofluorescence studies or immunoprecipitation methods), or in passive immunocontraceptive methods.

The present invention also includes transgenic non-human mammals, particularly murine and bovine mammals, that have been altered to contain a sequence which confers a deficiency in the normal expression of PT32 and/or c-Yes. Similarly, non-human transgenic mammals that overexpress PT32 and/or c-Yes are included within the invention. The mammals of the present invention can be heterozygous or homozygous for the desired trait, provided that the mammals contain the altered PT32 coding sequence.

As used herein, a mammal is said to be altered to contain a sequence which conveys a deficiency in the normal expression of PT32 if recombinant techniques are utilized to insert, delete or replace sequences encoding for, or directing the expression of, PT32. The insertion, deletion or replacement within such sequences has the effect of altering the normal level of expression of the given sequence or altering the activity of the protein which is expressed.

Mammals can be altered such that the mammal expresses a lower level of the protein when compared to a non-altered mammal (in some cases a mammal "deficient" in expressing normal levels of a protein will be incapable of expressing detectable levels of the given protein). In some instances, where a

mammal is altered such that a target gene is deleted or a large insertion is generated within the target sequence, the mammal will not produce detectable levels of the given protein. However, in some instances it may be possible for extremely low quantities of the protein to be produced, although such product may, in itself, be inoperative, or not functional in its usual physiological actions.

As used herein, "normal expression" is defined as the level of expression which is present in a wild-type or non-altered animal. A variety of techniques known in the art can be used to quantitate the level at which a given protein is expressed. These include, but are not limited to immunological techniques such as an ELISA, RIA, or western blot, or quantitative analytical techniques such as spectroscopy or flame chromatography.

Alternatively the mammals of the present invention can be altered so as to express an altered form of the given protein. Mammals can be altered such that a specific mutation is introduced into a given region of a PT32 protein.

The mammals of the present invention are preferably obtained by methods known in the art as homologous recombination (HR). This method has long been known in lower eukaryotes (e.g., yeast), and has also been described for the mouse (for review, see Capecchi, TIG 5(3):70-76 (1989) and also see Smithies *et al.*, *Nature* 317:230 (1985); Zijlstra *et al.*, *Nature* 342:435 (1989); Schwartzberg *et al.*, *Science* 246:799 (1989); DeChiara *et al.*, *Nature* 345:78 (1990)).

Homologous recombination essentially comprises isolating genomic sequences containing the target gene, employing known genetic engineering techniques to mutate or otherwise disable or modify the gene, and then reintroducing the gene into the relevant species. This is achieved by preparing a culture of pluripotent, or totipotent, cells, typically taken from embryos (ES cells). The advantage of these cells is that they can be successfully cultured for a large number of generations under conditions in which they will not differentiate and can be reintroduced into recipient embryos.

Typically the technique of electroporation, is used to render the ES cells capable of taking up exogenous DNA. The modified gene is then introduced, in a suitable manner, to these cells. Once taken up, recombination may occur,

although this may be by random integration as well as by homologous recombination.

To select cells in which a recombination event has taken place, a selectable marker sequence may be used. For example, it is well known to employ the bacterial Neo gene to confer resistance to neomycin, or an analogue thereof, such as G418. The marker gene may be inserted in the gene to be modified, thereby disabling the target gene, while providing a positive selectable marker. Clones which are Neo^+ have integrated the vector.

To further select homologous recombinants, the ends of the modified gene may have other markers inserted, such as the Herpes Simplex Virus thymidine kinase (HSVTK) gene. In a HR event, the HSVTK genes will not be recombined, and the marker will not be transferred. Therefore, the desired recombinant will be resistant to, for example, Gancyclovir, which is converted into a toxic metabolite when the HSVTK gene product is present (after a non-homologous recombination event).

Correct clones may be identified by the technique of PCR or by genomic Southern blotting. Subsequently, when a suitable clone has been identified, the ES cells may be injected into early-stage embryos, (blastocysts), and reintroduced into a pseudopregnant female. Chimeric animals will generally result from at least some of these embryos, their tissues deriving in part from the selected clone. Thus, the germ-line may also be chimeric, spermatozoa or ova containing the modified gene. Progeny deriving from such germ cells will be heterozygous for the gene. The heterozygous progeny can be cross-bred to yield homozygous animals. Confirmation of the allelic structure of the mammals can be ascertained by Southern blotting, for example.

The present invention also envisages cell lines suitable for generating mammals, particularly mice ,of the invention, and techniques for generating such lines and mice. Thus, to obtain mice according to the present invention, one skilled in the art can use the strategy of homologous recombination (HR) in embryonic stem cells (ES cells) to replace the wild-type sequences encoding PT32 with an altered sequence.

The absence of PT32 in a cell line or animal allows one skilled in the art to screen for genes and agents which can restore the altered mice to a wild-type phenotype, as well as to screen for agents which act as agonists or antagonists of PT32. Such animals are particularly useful as a source of abnormal spermatoza that can be used, for example, in studies of oocyte activation. In addition, such non-human mammals can be used as animal models in methods for treating humans. Additionally, the mammals of the present invention allow the investigation, at the cellular level as well as at the *in vivo* level, of a system which lacks PT32. This will allow researchers further to establish the importance of PT32. The animals and cells lines of the present invention may also be deficient in the expression of other genes, such as tyrosine kinase c-Yes, and thus provide the opportunity to study the interactions of PT32 and/or c-Yes with other proteins. Thus, it will be appreciated that there are many uses to which the mammals and cell lines of the present invention may be put. Artisans of ordinary skill will recognize that methods similar to the foregoing methods can be used to produce transgenic animals that are deficient in c-Yes expression, or to produce transgenic animals that overexpress PT32 and/or c-Yes.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples, certain methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accordance with conventional procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available, and their reaction

conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction may electrophoresed directly on a gel to isolate the desired fragment.

"Oligonucleotides" refers to either a single stranded polydeoxyribonucleotide or two complementary polydeoxyribonucleotide strands, which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate, e.g., with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., *et al.*, *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

"Oocyte activation" means the initiation of the resumption of second meiosis by an oocyte. Typically, oocyte activation is accompanied by induction of anti-polyspermy defense and pronuclear development. Oocyte activation begins with the cyclic release of calcium ions from the oocyte's endoplasmic reticulum (i.e., "calcium oscillations"). Thus, oocyte activation can be detected by detecting the release of calcium ions, e.g., using a conventional assay. Ultimately, oocyte activation typically leads to the first embryonic cleavage. *See, e.g.*, Perry *et al.*, *Developmental Biol. 217*:386-393 (2000), incorporated herein by reference.

Generally, techniques described herein can be performed essentially as described in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY

MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

The following examples illustrate the present invention and the advantages thereof. These examples are set forth to illustrate the invention, not limit the scope of the invention.

Examples

Gamete Preparation and In Vitro Fertilization of Bovine Oocytes with MitoTracker-tagged Sperm

Straws of frozen bull sperm (ABS, DeForest, WI) were thawed and centrifuged for 10 minutes at 700 x *g* through a two-layer (45 and 90%) Percoll gradient, then resuspended and incubated for 10 minutes at 37°C in a modified Tyrode's medium (Sperm-TL; Parrish, J. J., *et al., Theriogenology 25*:591-600 (1986); 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 29 mM NaH_2PO_4, 21.6 mM Na-lactate, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM HEPES, 6 mg/ml bovine serum albumin, 25 μg/ml gentamicin, 1 mM pyruvate), supplemented with 400 nM MitoTracker Green FM (Molecular Probes Inc., Eugene, OR), a vital, fixable mitochondrial dye with high affinity to sperm mitochondrial membranes (Sutovsky, P., *et al., Biol. Reprod. 55*:1195-1205 (1996)). MitoTracker-tagged sperm were washed by centrifugation in Sperm-TL.

Oocytes were isolated by aspiration from the ovaries obtained from a local abattoir (Walt's Meats Inc., Woodland, WA) and matured *in vitro* for 24 hours (metaphase II) in TC 199 medium (Gibco) supplemented with 10% fetal calf serum, 0.2 U/ml FSH-P (Schering-Plough, Kenilworth, NJ), 0.2 M pyruvate and 25 μg/ml gentamicin. Fertilization was performed according to the protocol of Parrish et al., (1986). Briefly, the MitoTracker-tagged sperm were resuspended in fertilization medium (TL; modified Tyrode-lactate medium: 114 mM NaCl, 3.2 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 10 mM Na-lactate, 6.5 i.u. penicillin, 25 μg/ml gentamicin, 6 mg/ml fatty acid-free bovine serum albumin and 0.2 mM pyruvate) supplemented with 0.25-5 μg/ml

heparin, and pipetted into 50 μl drops of fertilization medium with 10-15 mature oocytes/drop, to give a final concentration of 1 x 10^6 sperm/ml. Petri dishes with fertilization drops were incubated at 39°C in a humid atmosphere of 5% CO_2.

Immunofluorescence

The modified protocol of Sutovsky et al. (*Biol. Reprod.* 55:1195-1205 (1996)) was used to visualize sperm PT, nuclear pores, DNA and sperm tail mitochondria inside the zygotes. Fertilized oocytes were removed from the fertilization drops at various time points ranging from 8 hours (sperm incorporation) to 24 hours (first mitosis) after insemination and stripped of zonae pellucidae by 0.5% pronase in a serum-free TL-HEPES containing 0.5% polyvinyl-pyrolidone (PVP; Sigma), then attached to the poly-L-lysine coated microscopy coverslips in warm (37°C) 0.1 M phosphate-buffered saline (PBS; pH 7.2). Formaldehyde was added to the dishes with oocytes to a final concentration of 2% and fixed for 40 minutes at room temperature (RT). Zygotes were permeabilized overnight in 0.1 % Triton-X-100 (TX-100; Sigma) in 0.1 M PBS, blocked for 25 minutes with 5% normal goat serum (NGS; Sigma) in 0.1 M PBS with 0.1% TX-100 and incubated for 40 minutes at RT with a mixture of the perinuclear theca-specific rabbit polyclonal antibody pAb 427 (Oko, R., and Maravei, D., *Biol. Reprod. 50*:1000-1014 (1994); diluted (dil.) 1/200) and a nuclear pore-specific mouse monoclonal antibody mAb 414 (BabCo, Berkeley, CA; Davis, L. I., and Blobel, G., *Proc. Natl. Acad. Sci. USA 84*:7552-7556 (1987); Sutovsky, P., *et al., J. Cell Sci. 111*:2841-2854 (1998); dil., 1/200), or other antibodies, such as anti-PT32 antibodies, or anti-c-Yes antibodies (Santa Cruz Biotechnology), followed by a 40 minutes incubation with the red fluorescent, TRITC-conjugated goat anti-rabbit IgG and a far-red emitting, Cy5-conjugated goat anti mouse IgG (both from Zymed Labs, South San Francisco, CA; both dil. 1/40). DNA was stained by 4', 6'-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) added at 5 μg/ml to the secondary antibody solution 10 minutes before the end of incubation. All antibodies were diluted, and the zygotes were washed between and after the antibodies in 0.1 M PBS

containing 0.1% TX-100, 1% NGS and 0.05 NaN_3. The coverslips with zygotes were mounted on microscopy slides in a VectaShield mounting medium (Vector Labs, Burlingame, CA) and examined on a Zeiss Axiophot epifluorescence microscope equipped with an RTE/CCD 1217 camera (Princeton Instruments, Inc., Trenton, NJ), operated by MetaMorph software. Images were recorded onto Iomega Jazz cartridges (Iomega Corp., Roy, UT) and archived on recordable CDS. Due to its stability after formaldehyde cross-linking, the fluorescence of the Mitotracker-labeled sperm tail mitochondria was retained after such processing and allowed the discrimination between the male, sperm-tail tagged pronuclei, and female, tail-free pronuclei. Final images were created by pseudo-coloring and superimposing the parfocal single channel images using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA). Final composite images (PT=red, NPC=green, DNA=blue, sperm mitochondria=white) were contrast-enhanced, edited and printed on Sony UP-D-8800 color video printer using Adobe Photoshop 4.0. Two hundred and fifty zygotes and 50 oocytes were processed with the above antibodies and 50 zygotes were processed with preimmune rabbit serum as a negative control.

Preparation of the Perinuclear Theca Extracts

Isolated bull sperm heads were exposed to three successive extractions, consisting of incubations in 0.2% Triton X-100, 1M NaCl and 100mM NaOH. The first and second extractions solubilize the acrosome, head membranes, and hydrophobic and ionically bound proteins, leaving essentially a shell of insoluble perinuclear theca surrounding the condensed nucleus (Oko, R., and Maravei, D., *Biol. Reprod. 50*:1000-1014 (1994)). Subsequent extraction with NaOH solubilizes the PT but leaves the nucleus in its condensed form. The supernatant recovered in this last extraction step (PT extract) was neutralized, dialyzed and lyophilized for use in SDS-PAGE analysis and western blotting as described previously by Oko and Maravei (1994) and, for microinjection into the egg. All the antibodies raised against this PT extract (see above reference), whether raised against the whole extract (pAB 427) or against each of its major proteins,

exclusively immunolocalized to the PT of the sperm head, providing the confidence for the specificity of this extraction technique.

Intracytoplasmic Injection of Perinuclear Theca Extracts

Mature oocytes, selected for micro injection, were placed in a 100 ml Ca^{2+}-free TALP-HEPES medium, under oil, on the stage of a Nikon Diaphot inverted microscope. Each oocyte was immobilized using a Narishigi holding pipette with the polar body at 12 o'clock. A calibrated injection pipette attached to an Eppendorf motorized manipulator was used to deliver 20 pl PT extract into each oocyte. Control oocytes were injected with the same volume of Ca^{2+}-free TALP-HEPES medium. Injected oocytes were cultured in fertilization medium until fixation at 12 hours post injection.

SDS-PAGE and Western Blotting

Lyophilized PT extracts were solubilized in 2% SDS, 5% β-mercaptoethanol by boiling for 5 minutes and then run on linear gradient (8-18%) polyacrylamide gels according to the SDS-discontinuous system originally described by Laemmli, U. K., *Nature 277*:680-685 (1970). Preparative gels were eletrophoretically transferred to nitrocellulose (Schleicher and Schuell Inc., Keene, NU) utilizing a Hoefer Wet Transphor apparatus according to the technique of Towbin, H., and Gordon, J., *J. Immunol. Meth. 72*:313-340 (1984). The immuno-reactivity of western blotted proteins to pAB 427 was detected by developing the phosphatase color reaction on the secondary antibody phosphatase conjugate (alkaline phosphatase conjugated $F(ab)^2$ goat anti-rabbit IgG; Cappel-Cooper Biomedical Inc.,Malvern, PA) according to McGadey, J., *Histochemie 23*:180-184 (1970).

Transmission Electron Microscopy

Fertilized oocytes were fixed in formaldehyde-glutaraldehyde fixative of Ito, S., and Karnovsky, M. J., *J. Cell Biol. 89*:168a (1968), containing 5% 2-4-6 trinitrophenol (picric acid; Sigma), post-fixed in 1% osmium tetroxide, dehydrated by an ascending ethanol series (30-100%), perfused with the solution of acetone and Epon 812, and embedded in Epon 812 resin. Tissue sections were cut using a Sorvall MT 5000 ultramicrotome, transferred onto 100 MESH Cu-grids, stained with uranyl acetate and lead citrate, and examined and photographed on a Philips 300 electron microscope. Negatives were scanned by an Umax Power Look 3000 scanner and printed using Adobe Photoshop 4.0 software.

Perinuclear Theca-Oolemma Binding Assay

Bull sperm were processed as described above and deprived of their plasma membranes by a 20 minutes incubation at 37°C in 0.05% lysophosphatidyl-choline (Lysolecithin; Sigma) diluted in the KNIT medium (100 mM KCl,2 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.0). Mature, metaphase-II-arrested live oocytes were deprived of zonae pellucidae as described for immunofluorescence and transferred into drops of fertilization medium. One million demembranated sperm/ml were mixed with zona free oocytes, cultured for 4 hours, and fixed for immunofluorescence (pAb 427/DAPI, or DAPI only) or electron microscopy as described above.

Results

Incorporation of the Sperm Perinuclear Theca Into Oocyte Cytoplasm During Natural Fertilization

Labeling of the fertilized oocytes with the PT-specific antibody pAb 427 was combined with the NPC-specific antibody mAb 414 and the DNA stain DAPI, and with the use of MitoTracker-tagged spermatozoa, in order to monitor the removal and incorporation of sperm PT at fertilization. With the exception of DAPI-stained maternal chromosomes, the unfertilized, metaphase II-arrested

oocytes displayed neither of the above immunofluorescent labelings (Fig. 3A). The intact PT was found on the surface of the oocyte-bound spermatozoa at an early stage of fertilization (Figs. 3B, C), and the PT-labeling acquired fuzzy appearance in the spermatozoa undergoing the incorporation into oocyte cytoplasm (Fig. 3D). Clumps of the PT-derived material were found next to the incorporated sperm nuclei when the new nuclear envelope, delineated by the mAb 414-positive NPCs, reformed on their surface (Fig. 3E). Such clumps of PT-material were seen near the male pronucleus throughout the initial stages of pronuclear development, during which the female chromatin completed second meiotic division and the oocytes extruded the second polar bodies (Fig. 3F-H). Remnants of the PT were last seen in the zygotes in which the developing male and female pronuclei entered the process of pronuclear apposition (Fig. 3I). No PT-derived material was detected in the zygotes reaching full pronuclear size (Fig. 3J, K) and apposition (Fig. 3L, M), and in the spontaneously activated parthenogenetic oocytes (Fig. 3N). Such spontaneous parthenogenotes also displayed aberrant patterns of NPC assembly (Fig. 3N).

Oocyte Activation Induced by the Intracytoplasmic Microinjection of Purified Perinuclear Theca-Extracts

The hypothesis that the material released from sperm PT induces the activation of bovine oocytes was initially tested using ICSI of the intact spermatozoa. In bovine, this method yields very moderate activation and pronuclear development rates, thus requiring artificial activation (*e.g.*, Rho, G.-J., *et al., Biol. Reprod. 59*:918-924 (1998)). In our experiments, which did not include chemical activation, 24 out of 27 sperm-injected oocytes remained in metaphase-II 20 hours after ICSI, and the intact spermatozoa in their cytoplasm displayed intense labeling with pAb 427 in the subacrosomal region (Fig. 4A, B) and on the equatorial segment (Fig. 4C). Three out of 27 oocytes displayed certain signs of activation after ICSI. In one case, the PT-derived material was released from the sperm head into surrounding cytoplasm and the maternal chromatin displayed signs of decondensation (Fig. 4D). Decondensation of both the paternal and the maternal chromatin was seen in an oocyte in which the sperm

nucleus contained two separate clumps of PT material (Fig. 4E). Finally, one oocyte developed a normal female pronucleus surrounded by the NPC-containing nuclear envelope (Fig. 4F). In this oocyte, the sperm PT was still detectable on the equatorial segment of the sperm nucleus, but completely absent from the subacrosomal region (Fig. 4F).

Injection of PT extracts induced oocyte activation, accompanied by the formation of one (Fig. 4G) or two (Fig. 4H) female pronuclei, in 27.7% of oocytes. These oocytes displayed typical dot-like pattern of AL/NPC assembly in the cytoplasm (Sutovsky, P., *et al., J. Cell Sci. 111*:2841-2854 (1998)), and a fing of NPCs on their nucleus (Fig. 4G, H). Those oocytes that failed to activate after the injection of PT extracts, displayed a metaphase-like arrangement of female chromosomes and their cytoplasm was free of AL (Fig. 4I). The boiling of the extracts lowered the rate of oocyte activation to a level seen in the sham-injected group. Two out of 14 (12.5%) of oocytes in one experiment became activated after the micro injection of boiled PT-extracts (Fig. 4J). Sham injection of a calcium-free culture medium failed to activate 88.6% of the injected oocytes (Fig. 4 K). One or two pronuclei were formed in the oocytes that became activated after sham-injection (11.4%), yet those oocytes did not support the cytoplasmic assembly of NPCs into AL and displayed the aberrant formation of pronuclear NPCs (Fig. 4L, M).

Protein Composition of Perinuclear Theca-Extracts Used for Micro injection Into Oocyte Cytoplasm

Perinuclear theca. extracts used in the above micro injection experiments were composed of five major proteins of 15-, 25-, 28-, 32-, and 60- kDa as (Fig. 5A), all of which were previously immuno-localized to the PT (Oko, R., and Maravei, D., *Biol. Reprod. 50*:1000-1014 (1994)). These proteins were transferred onto nitrocellulose and probed with rabbit polyclonal antibody pAb 427 that only labels the PT in sperm prepared for immunocytochemistry (Oko, R., and Maravei, D., *Biol. Reprod. 50*:1000-1014 (1994); Sutovsky, P., *et al., Dev. Biol. 188*:75-84 (1997a)). Depending on which boost of this immune serum was used, the transferred proteins immuno-reacted with varying intensities. With

the first, second and third boosts the 15- and 28-, 32-, and 36-kDa bands, respectively, were the most reactive of the major PT proteins (Fig. 5B). These proteins, which are designated with a prefix PT to reflect their origin in perinuclear theca (e.g., PT 15, PT 32).

Ultrastructure of the Interactions Between Sperm Perinuclear Theca and Oocyte Cortex

Despite the fact that the process of sperm incorporation occurs rapidly and is seldom documented by electron microscopy, we succeeded in observing the interactions between sperm PT and the oocyte cortex/cytoplasm in several specimens. During sperm-oolemma binding, the oocyte microvilli seemed to fuse with sperm plasma membrane and this new zygotic membrane remained attached to the sperm PT (Fig. 6A, B). Such binding of PT to oolemma was also observed during sperm incorporation into oocyte cytoplasm, when the oocyte microvilli remained attached to the innermost leaf of PT, while the outer layers of PT became detached from the sperm nucleus and partially dissolved in the cytoplasm (Fig. 6C). The dissolution of the sperm PT in the oocyte cytoplasm was also found in a partially incorporated spermatozoon, the complete incorporation of which was prevented by polyspermy block (Fig. 5D-F). The cytoplasm of this oocyte contained one female and one sperm tail-tagged male pronucleus with no signs of PT (not shown). The post-acrosomal sheath of the head of second spermatozoon, protruding into the perivitelline space, contained an intact PT (Fig. 6D-F). In contrast, the incorporated apical segment of this sperm head contained no PT and displayed partially decondensed chromatin surrounded by a newly formed nuclear envelope (Fig. 6D-F). To support the observations on PT-oolemma binding, bull spermatozoa were demembranated with 0.05% lysolecithin to expose their PT and co-cultured for 16 hours with zona-free oocytes. This treatment resulted in a strong binding of oocyte microvilli to the PT of such spennatozoa (Fig. 6G-I).

These experiments indicate that the perinuclear theca of mammalian spermatozoa contains factor(s) capable of triggering oocyte activation at fertilization. Supportive of such a role for PT are the studies in the

globozoospermic infertility patients, whose spermatozoa lack PT and fail to induce oocyte activation after ICSI (Battaglia, D. E., *et al., Fertil. Steril. 68*:118-122 (1997); Rybouchkin, A., *et al.,. Hum. Reprod. 11*:2170-2175 (1996); Rybouchkin, A. V., *et al., Fertil. Steril. 68*:1144-1147 (1997)). Furthermore, crude whole sperm extracts (Swann, K., *Development 110*:1295-1302 (1990)) and those prepared from the isolated sperm heads (Kimura, Y., *et al., Biol. Reprod. 58*:1407-1415 (1998); Perry, A. C. F., *et al., Biol. Reprod. 60*:747-755 (1999)) induced oocyte activation after extract micro injection in rodents. We have used a three step extraction of isolated sperm heads to obtain pure extracts of the bull sperm PT. By carefully checking the outcome of each step by electron microscopy, we visually eliminated the possibility that proteins of other sperm head components would contaminate such extracts. In addition, all antibodies raised against whole PT extracts, including pAB427, exclusively label the PT of sperm prepared for immunocytochemistry (Oko, R., and Maravei, D., *Biol. Reprod. 50*:1000-1014 (1994)) confirming the purity of the preparation. When the PT extracts prepared for this study were subjected to electrophoresis and Western blotting, the Coomassie blue-stained bands overlapped with those cross-reacting with the PT-specific polyclonal antibody pAb 427, with the exception of the non-reactive major 60 kDa band which we have previously identified as PT 60 (Oko, R., and Maravei, D., *Biol. Reprod. 50*:1000-1014 (1994)).

Use of PT32 in Mammalian Oocyte Activation

Our further efforts have focused on the isolation and characterization of individual PT proteins with oscillogenic activity, particularly the 32 kDa polypeptide (PT32), which is found in the PT of sperm of several mammalian species including bovine, mouse, and human. A polyclonal anti-PT32 antibody was used to screen a bull testicular cDNA expression library, and 5 positive cDNA clones having similar sequence identity were isolated. The longest sequence obtained was 1413 nt in length, and included an open reading from of 939 nt, encoding a protein of 313 amino acids. The nucleic acid and amino acid sequences of an exemplary PT32 protein are set forth in Fig. 1. This PT32

protein has a calculated molecular weight of 31,964 Da (i.e., about 32kDa). The deduced amino acid sequence shares 58% homology with WW domain Binding protein 2 (WBP2). PT32 shares N-terminal sequence similarity and proline rich motifs with the WBP2, which binds to the WW domain of *Yes*-associated protein in the *src* family tyrosine kinase cascade (Chen, H. I., and Sudol, M., *Proc. Natl. Acad. Sci. USA 92*:7819-7823 (1995); Sudol, M., *et al., FEBS Lett. 369*:67-71 (1995); Sudol, I. M., *Oncogene 17*:1469-1474 (1998)).

The assembly of cytoplasmic annulate lamellae and nuclear pore complexes are two typical indicators of sperm-induced oocyte activation in mammals (Sutovsky, P., *et al., J. Cell Sci. 111*:2841-2854 (1998)). We observed these events in the oocytes activated by micro injection of PT extracts, but not in those activated spontaneously or after the sham-injection of culture medium. Abnormal patterns of NPC and AL assembly were also seen in bovine oocytes activated by the combination of actinomycin and 6-dimethyl-amino-purine, a treatment most commonly used for artificial activation in animal ART protocols. In addition to the cortical reaction and the onset of calcium oscillations, oocyte activation in mammals apparently typically encompasses the rearrangement of cytoplasmic organelles and membranes. Various artificial activation stimuli may induce these changes to various degrees. A natural stimulus, such as the release of oscillogenic factor(s) from the isolated sperm PT, or treatment with a recombinant PT32 protein, and, optionally, recombinant c-Yes, is thus a preferred means of oocyte activation in both human and animal ART protocols.

The experiments disclosed herein demonstrates that, after being detached from the sperm nucleus, the PT is incorporated into oocyte cytoplasm and completely dissolved in it before the pronuclei reach their full size and become apposed. Furthermore, the segments of the sperm PT exposed to oocyte cytoplasm dissolve in it even if the complete entry of the sperm head into the oocyte cytoplasm is prevented by a polyspermy block (as described above). The PT-mediated introduction of the sperm head-anchored oscillogens into oocyte cytoplasm (see Fig. 7) helps explain the sperm-induced oocyte activation in mammals without the need for hypothetic large, activation-permissive pores in the oolemma (Jones, K. T., *et al., Development 125*:4627-4635 (1998)).

The foregoing examples demonstrate that the sperm perinuclear theca, after being detached from the sperm nucleus, becomes incorporated into oocyte cytoplasm and completely dissolved prior to the completion of pronuclear development and apposition. Injection of isolated PT extracts into the unfertilized oocytes induces their activation, accompanied by the cytoplasmic and nuclear events that mirror natural fertilization. We conclude that sperm PT anchors the oocyte activating factor(s) and the PT's partial or complete incorporation into oocyte cytoplasm is the natural mechanism by which the fertilizing spermatozoon activates the oocyte.

Involvement of PT32 and Tyrosine Kinase c-Yes in the Activation of Mammalian Oocytes

The experiments set forth below demonstrate that the sperm perinuclear theca protein PT32 interacts with tyrosine kinase c-Yes and induces activation of mammalian oocytes. As illustrated in Fig. 1, the amino acid sequence of PT32 contains 12 repeats of an unique protein binding domain and a consensus site for binding to the WW-module of the Yes-binding protein in the signaling cascade of the Src-family, non-receptor tyrosine kinase c-Yes. An isoform of c-Yes is present in the oocyte cytoplasm, and in sperm PT starting with the elongated spermatid, a stage at which spermatids acquire the ability to induce oocyte activation. As demonstrated below, the injection of the recombinant PT32 (rPT32) into bovine oocytes causes the clustering of c-Yes in the oocyte cytoplasm, and causes oocyte activation at rates comparable to those of oocytes injected with detergent-insoluble PT-extracts. These data are consistent with the interaction of sperm PT32 and c-Yes, and the oocyte cytoplasmic c-Yes, in oocyte activation.

Oocyte activation typically encompasses the resumption of second meiosis by the oocyte, induction of anti-polyspermy-defense, and pronuclear development. These events begin with the cyclic release of Ca^{2+} ions from oocyte's endoplasmic reticulum (ER), also referred to as calcium oscillations, and ultimately lead to the first embryonic cleavage. The data presented herein

provide evidence for the involvement of the major PT protein, PT32, and the PT-sequestrated isoform of Src family protein tyrosine kinase c-Yes (Summy et al., 2000) in oocyte activation. PT32 contains a consensus binding site for the Yes kinase-binding protein. In contrast to previous studies using crude, soluble sperm extracts generated by repeated freezing/thawing procedures (Swann, 1996), sometimes complemented with DTT-solubilized sperm head factors (Perry et al., 2000), recombinant PT 32 (rPT32) and purified PT extracts obtained by alkaline extraction (Oko and Maravei, 1994; 1995) were used for the microinjection experiments described herein.

Antibodies used in the experiments described below were raised against portions of PT32, which were designated oligopeptide regions 1 and 2 and shown in bold in Fig.1. These oligopeptides included the amino acid sequences TSYRVVFVTSHLVNDPMLSFMMPF (SEQ ID NO:6) and NEALPPAYEAPSAGNT (SEQ ID NO:7).

In a control experiment, using a fertilization medium (FM) that supports early zygotic, but not embryonic bovine development, rPT 32, perinuclear theca extract (PTX) and bovine serum albumin (BSA-V; control protein) were injected, separately, into the cytoplasm of the metaphase-II arrested oocytes, which was cultured for 40 hours after injection (Fig. 8A). Only a slight increase in cleavage rates was observed in rPT32-injected oocytes, as compared with the BSA-V group, while the total rate of activation, including pronuclear development rates, was not significantly different among individual groups, probably due to the spontaneous pronuclear development after prolonged culture in FM. This can probably be attributed to the effect of egg plasma membrane damage by the injection alone, and to the spontaneous cessation of protein kinase activity, necessary to maintain metaphase-II arrest, during egg aging in culture medium (Eyestone and Campbell, 1999).

To demonstrate the ability of rPT32 to induce oocyte activation, rPT 32, perinuclear theca extracts (PTX), and bovine serum albumin (BSA-V; control protein) were injected, separately, into the cytoplasm of the metaphase-II arrested oocytes under enhanced culture conditions. Injected oocytes were cultured in FM for 24 hours and further cultured in the medium CR-1, which supports embryonic

development (Fig. 8B). Both pronuclear development and cleavage rates are significantly higher in groups injected with rPT32 or PTX, as opposed to oocytes that were sham-injected with BSA-V or oocytes cultured for 40 hours under identical conditions but without injection. Heat treatment (boiling for 20 minutes) did not substantially affect the ability of PT extracts (PTX boiled) to activate the oocytes, and actually increased the cleavage rate.

To demonstrate the interaction of rPT32 with c-Yes, rPT32 or PT extracts were injected into oocytes. PT32 induced the clustering of intrinsic c-Yes kinase in the oocyte cytoplasm at rates comparable to those of oocytes injected with whole PT extracts (Figs. 9A-4F). Oocytes were fixed at metaphase-II prior to microinjection (Fig. 9A), 20 hours after injection of rPT 32 (Fig. 9B), crude PT extracts (Fig. 9C), BSA-V (Fig. 9D), or after 20 hours of culture in fertilization medium, without injection (Fig. 9E). Figs. 9F and 9G illustrate the partial overlap of c-Yes speckles (red) with endoplasmic reticulum marker α-PDI (green). Fig. 9H illustrates the occurrence of c-Yes-positive speckles in the cytoplasm of oocytes injected as described above, as demonstrated by subjective evaluation after immunofluorescence labeling. Representative images of oocytes with low, medium, and high clustering of c-Yes are shown in Figs. 9A, B and C, respectively. In Fig. 9B, the left oocyte displays medium clustering, and the right oocyte displays high clustering of c-Yes. Spindle and midbody microtubules (green in Figs. 9 A, B) were labeled with the anti-β-tubulin antibody E7, DNA (blue in Figs. 9 A-F) was stained with DAPI. Thus, these experiments provide evidence that PT32 interacts (directly or indirectly) with c-Yes.

Figs. 10A-5F illustrate the pattern of expression of c-Yes (Figs. 10A and 10B), PT15, which is a histone-like PT protein without known signaling motifs(Fig. 10C) and PT 32 (Figs. 10D-F) in sperm and spermatids of bull. PT32 was detected using the peptide-specific antibodies raised against oligopeptide regions 1 and 2, as described above. Note that c-Yes (Fig. 5A), but not PT 15 (Fig. 10B) and PT32 (Fig. 10C) is absent from round spermatids (arrows in Figs. 10A, 10C, and 10D), and becomes inserted into PT during spermatid elongation. Note the diminished cross-reactivity of anti-c-Yes antibody in ejaculated sperm

(Fig. 10B). All PT proteins are shown in green; DNA was stained with DAPI and is shown in blue.

In summary, the foregoing examples illustrate that PT32 interacts with tyrosine kinase c-Yes and induces activation of mammalian oocytes.

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What Is Claimed Is:

1. An isolated polypeptide comprising

(a) at least one of (i) the sequence PPPGY (SEQ ID NO: 1) and (ii) the sequence LPPAY (SEQ ID NO: 2) and

(b) at least three domains, each domain comprising the sequence YGXPPXG (SEQ ID NO:3), wherein Y represents a Tyrosine residue, G represents a Glycine residue, L represents a Leucine residue, A represents an Alanine residue, X represent any amino acid residue, and P represents a Proline residue.

2. The isolated polypeptide of claim 1, wherein the polypeptide has a molecular weight of about 32 kDa.

3. The isolated polypeptide of claim 1, wherein the polypeptide comprises 10 domains, each domain comprising the sequence YGXPPXG (SEQ ID NO:3).

4. The isolated polypeptide of claim 1, wherein the polypeptide binds to tyrosine kinase c-Yes.

5. The isolated polypeptide of claim 1, wherein the polypeptide induces oocyte activation.

6. The isolated polypeptide of claim 1, wherein the polypeptide comprises the sequence of SEQ ID NO: 5, as illustrated in Fig. 1, or conservative variants thereof.

7. A peptidomimetic of the polypeptide of claim 1.

8. An isolated polynucleotide encoding the polypeptide of claim 1.

9. An isolated polynucleotide encoding the polypeptide of claim 6.

10. The isolated polynucleotide of claim 8, wherein the polynucleotide comprises the sequence of SEQ ID NO: 4, as illustrated in Fig. 1, or degenerate variants thereof.

11. A gene comprising the polynucleotide of claim 8.

12. A vector comprising the gene of claim 11.

13. A vector comprising the polynucleotide of claim 8.

14. A host cell comprising the vector of claim 12.

15. A method of producing a polypeptide, the method comprising maintaining the host cell of claim 14 under conditions such that said polypeptide is expressed, then collecting the polypeptide.

16. A fragment of the polypeptide of claim 1, wherein the fragment is antigenic.

17. A fragment of the polypeptide of claim 1, wherein the fragment is biologically active.

18. A fragment of the polypeptide of claim 1, wherein the fragment binds to tyrosine kinase c-Yes.

19. An antibody that specifically binds to the polypeptide of claim 1.

20. The antibody of claim 19, wherein the antibody is a monoclonal antibody.

21. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the antibody of claim 19.

22. A pharmaceutical composition comprising a pharmaceutically acceptable foam and at least one molecule selected from the group consisting of an antibody that specifically binds to PT32, an antibody that specifically binds to c-Yes, PT32 or a fragment thereof, c-Yes or a fragment thereof, an agonist or antagonist of PT32, and an agonist or antagonist of c-Yes.

23. A method for inducing oocyte activation, the method comprising contacting an oocyte with at least one of (i) the isolated polypeptide of claim 5 or a biologically active fragment thereof, (ii) a c-Yes polypeptide or a biologically active fragment thereof, and (iii) globozoospermic sperm.

24. A method for inducing oocyte activation, the method comprising contacting an oocyte with a composition consisting essentially of a perinuclear theca protein 32 (PT32), or a biologically active fragment thereof.

25. A method for enhancing fertility in a mammal, the method comprising expressing in a germ cell of the mammal the polypeptide of claim 5.

26. The method of claim 25, wherein the mammal is a human.

27. The method of claim 25, wherein the mammal is bovine, a pig, a sheep, a goat, a monkey, or a horse.

28. A method for treating globozoospermy, the method comprising expressing in spermatoza the polypeptide of claim 1.

29. A method for identifying a modulator of oocyte activation, the method comprising

contacting a test compound with an oocyte,

treating the oocyte with the polypeptide of claim 5 under conditions sufficient to induce oocyte activation in the absence of the test compound, and

detecting inhibition or enhancement of oocyte induction as an indication that the test compound is an modulator of oocyte activation.

30. A method for identifying a modulator of oocyte activation, the method comprising

contacting a test compound with (i) the polypeptide of claim 1, or a tyrosine kinase c-Yes-binding fragment thereof, and (ii) tyrosine kinase c-Yes, or a PT32-binding fragment thereof, under conditions sufficient to permit binding of the polypeptide of claim 1 or the tyrosine kinase c-Yes-binding fragment thereof to tyrosine kinase c-Yes or to the PT32-binding fragment thereof in the absence of the test compound, and

detecting modulation of binding of the polypeptide of claim 1, or the fragment thereof, to the tyrosine kinase c-Yes, or the fragment thereof, as an indication that the test compound is an modulator of oocyte activation.

31. A method for inhibiting fertilization of a mammalian oocyte, the method comprising inhibiting the interaction of PT32 with tyrosine kinase c-Yes in the oocyte.

32. The method of claim 31, wherein inhibition comprises contacting the oocyte with at least one of (i) an antibody that specifically binds to PT32 and (ii) an antibody that specifically binds to tyrosine kinase c-Yes.

33. A method for inhibiting fertilization, the method comprising introducing into a mammal the polypeptide of claim 1, or an antigenic fragment thereof, such that an immune response is elicited in the mammal.

34. A fusion polypeptide comprising the polypeptide of claim 1, or a fragment thereof, covalently linked to a second polypeptide.

35. A vaccine comprising the fusion polypeptide of claim 34 and an adjuvant.

36. A vaccine comprising the polypeptide of claim 16 and an adjuvant.

37. A method for diagnosing diminished fertility in a mammal, the method comprising measuring the level of the polypeptide of claim 1 in spermatoza of the mammal, wherein diminished levels of the polypeptide of claim 1 indicate that the mammal suffers from diminished fertility.

38. A method for diagnosing diminished fertility in a mammal, the method comprising measuring the level of tyrosine kinase c-Yes in a germ cell of the mammal, wherein diminished levels of tyrosine kinase c-Yes indicate that the mammal suffers from diminished fertility.

39. A method for diagnosing abnormal spermiogenesis in a mammal, the method comprising comparing (i) the pattern of the distribution of the polypeptide of claim 1 throughout mature spermatoza of the mammal with (ii) the pattern of the distribution of the polypeptide of claim 1 throughout healthy, mature spermatoza, wherein an abnormal distribution pattern is an indication that spermiogenesis in the mammal is abnormal.

40. A method for diagnosing abnormal spermiogenesis in a mammal, the method comprising comparing (i) the pattern of the distribution of tyrosine kinase c-Yes throughout mature spermatoza of the mammal with (ii) the pattern of the distribution of tyrosine kinase c-Yes throughout healthy, mature spermatoza, wherein an abnormal distribution pattern is an indication that spermiogenesis in the mammal is abnormal.

41. A method for determining whether spermiogenesis is abnormal in a mammal, the method comprising determining the pattern of the distribution of PT32 throughout spermatoza of the mammal, wherein the failure of the PT32 to

be localized either (i) between the acrosome and the nucleus of the spermatoza or (ii) on a post-acrosomal portion of the head of the spermatoza is an indication that spermiogenesis is abnormal in the mammal.

42. A transgenic non-human mammal whose germ cells contain a disruption in the endogenous gene encoding PT32, wherein said disruption comprises the insertion of a selectable marker sequence, and wherein said disruption results in the lack of expression or function of said PT32.

43. The transgenic mammal of claim 42, wherein the mammal is a mouse or monkey.

44. A method for enhancing oocyte activation, the method comprising contacting a mammalian oocyte with tyrosine kinase c-Yes, or a biologically active fragment thereof.

45. A transgenic non-human mammal whose germ cells contain a disruption in the endogenous gene encoding tyrosine kinase c-Yes, wherein said disruption comprises the insertion of a selectable marker sequence, and wherein said disruption results in the lack of expression or function of said tyrosine kinase c-Yes.

PT32 Sperm Protein, Sperm c-Yes, Oocyte Cytoplasmic c-Yes, and Uses Thereof

Abstract

The perinuclear theca 32 (PT32) protein is disclosed and shown to interact with tyrosine kinase c-Yes. PT32, c-Yes, fragments thereof, and molecules that bind thereto can be used in methods of enhancing fertility, treating or diagnosing diminished fertility and abnormal spermiogenesis, in providing contraception, and in identifying contraceptive and fertility-enhancing agents. Transgenic, non-human animals also are disclosed.

```
   1 GCACGAGGGGCGGCAGGAGGGGGCCTGGGCAGGATGGCAGTGAACCAGAGCCACACCGAG
   1  A  R  G  A  A  G  G  G  L  G  R  M  A  V  N  Q  S  H  T  E
  61 AGCCGTCGTGGGGCCCTCATCCCCTCTGGCGAAAGTGTCTTGAAGCAGTGTGAGGATGTG
  21  S  R  R  G  A  L  I  P  S  G  E  S  V  L  K  Q  C  E  D  V
 121 GACCTCTGCTTCCTACAGAAACCAGTGGAATCCTATCTCTTTAATGGCACAAAGAAAGGA
  41  D  L  C  F  L  Q  K  P  V  E  S  Y  L  F  N  G  T  K  K  G
 181 ACGTTGTTTCTCACTTCATACCGGGTGGTCTTCGTGACTTCACACTTAGTCAATGACCCC
  61  T  L  F  L  T  S  Y  R  V  V  F  V  T  S  H  L  V  N  D  P  <1.
 241 ATGCTTTCTTTTATGATGCCGTTTGGCCTGATGAGTGACTGCACCATTGAACAACCAATT
  81  M  L  S  F  M  M  P  F  G  L  M  S  D  C  T  I  E  Q  P  I
 301 TTTGCCCCCAACTACATTAAAGGAACCATTCAGGCAGCTCCAGGTGGTGGCTGGGAAGGA
 101  F  A  P  N  Y  Y  I  K  G  T  I  Q  A  A  P  G  G  G  W  E  G
 361 CAAGCTGTTTTTAAGTTATCCTTCAGGAAAGGAGGTGCCATCGAATTTGCCCAACTGATG
 121  Q  A  V  F  K  L  S  F  R  K  G  G  A  I  E  F  A  Q  L  M
 421 GTAAAAGCTGCCTCTGCTGCTGCCAGAGGAATTCCACTTGGAAGTGTAAATTACTGGTTC
 141  V  K  A  A  S  A  A  A  R  G  I  P  L  G  S  V  N  Y  W  F
 481 GACACTTCAGGACTGTACATAATTACTGTCCCAGGGGCTGCAGTGTGCTCCTCACAGACA
 161  D  T  S  G  L  Y  I  I  T  V  P  G  A  A  V  C  S  S  Q  T
 541 CCTTGTCCAGCATATCCAATTGTGATCTATGGACCCCCACCACCAGGATATACAGTCCAA
 181  P  C  P  A  Y  P  I  V  I  Y  G  P  P  P  P  G  Y  T  V  Q
 601 CCAGGGGAATATGGAACTCCACCAGAAGGATATGGAGCCCAACCAGGGGGATATGGAGCC
 201  P  G  E  Y  G  T  P  P  E  G  Y  G  A  Q  P  G  G  Y  G  A
 661 CCACCTATGGGATATGGAGCCCCGCCTGTGGGATATGGAGTCCCACCTGGGGGATATGGA
 221  P  P  M  G  Y  G  A  P  P  V  G  Y  G  V  P  P  G  G  Y  G
 721 GTCCCACCTGGGGGATATGGAGTCCCACCTGGGGGATATGGAGCCCCACCTGGGGGATAT
 241  V  P  P  G  G  Y  G  V  P  P  G  G  Y  G  A  P  P  G  G  Y
 781 GGAGTCCCACCTGGGGGATATGGTGCCCCACCTGGGGGATATGGAGCCCCACCTGCAGGA
 261  G  V  P  P  G  G  Y  G  A  P  P  G  G  Y  G  A  P  P  A  G
 841 TATGGAGCCCCACCAGCTGGAAATGAAGCCCTACCCCCTGCATATGAAGCTCCATCTGCT
 281  Y  G  A  P  P  A  G  N  E  A  L  P  P  A  Y  E  A  P  S  A  <2.
 901 GGAAATACAGCTGCCTCTCACAGATCTATGACAGCTCAGCAGGAGACTTCTCTTCCCACT
 301  G  N  T  A  A  S  H  R  S  M  T  A  Q  Q  E  T  S  L  P  T
 961 ACCTCATCTTCTTAGGTCCATTTACCACCTTCTCAGAGTTAAACCTTGAAGACTCACCAA
 321  T  S  S  S  •••
1021 GCAAAGGGCACCCTAAAACTGAAGTCACAGTAAGAAGGAAGACCCAGGTGCCCAGTGGTA
1081 GGAGGTGTTCGTGTGCACGCAGTGGTCTGATCTTCTCCACACACCTGTGAGGTCCTGTGC
1141 CTCAAAACAGATGAAGGTGAGAAGACGACTCCTGTTCTCAAGGAAGGAAGATGCTTGAAA
1201 ACAGACTGCAAGCCAACTAGAGAGAGAGAGATGTGAAGTGGCACATAAAACAGCTTGGGG
1261 ATGGAGACTGACTCTCTTTAGAAAACAGGCCTTCTCCCTGCCTCTGACCTGAGCAGAAAA
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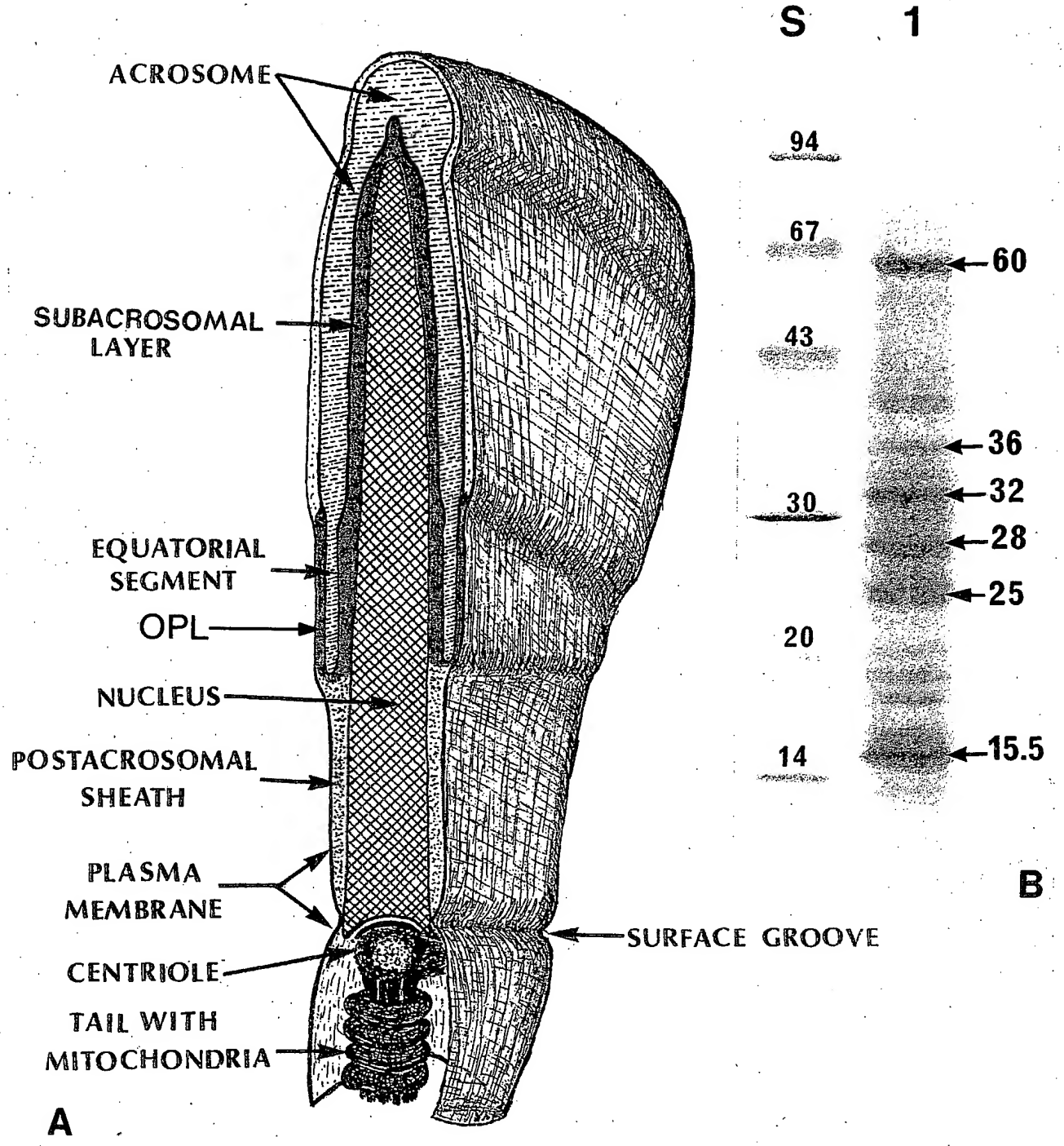
FIG 1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SUBACROSOMAL LAYER
EQUATORIAL SEGMENT
OPL
NUCLEUS
POSTACROSOMAL SHEATH
PLASMA MEMBRANE
CENTRIOLE
TAIL WITH MITOCHONDRIA
A
SURFACE GROOVE
S
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94
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43
30
20
14
60
36
32
28
25
15.5
B

FIG 2

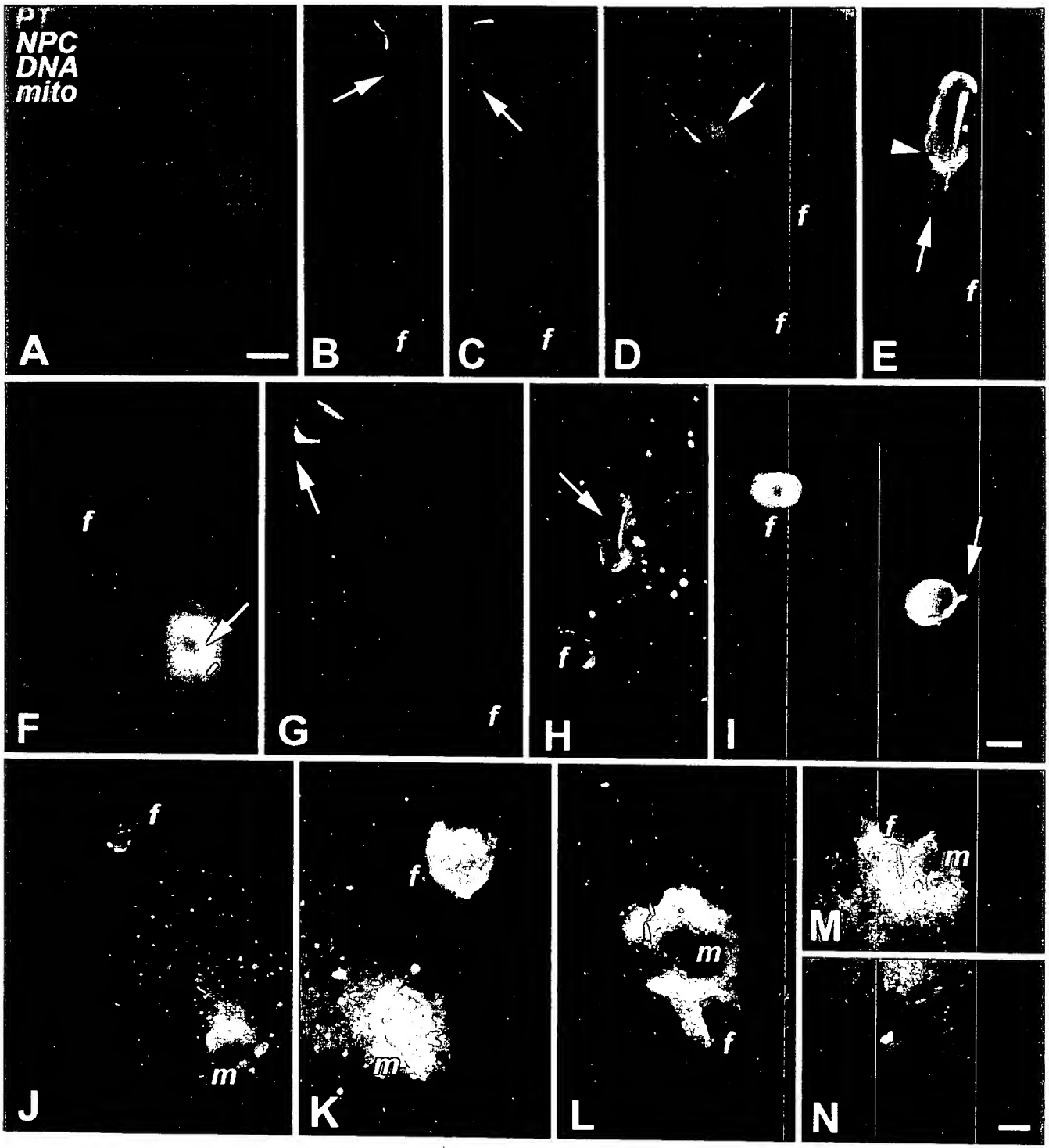


FIG 3

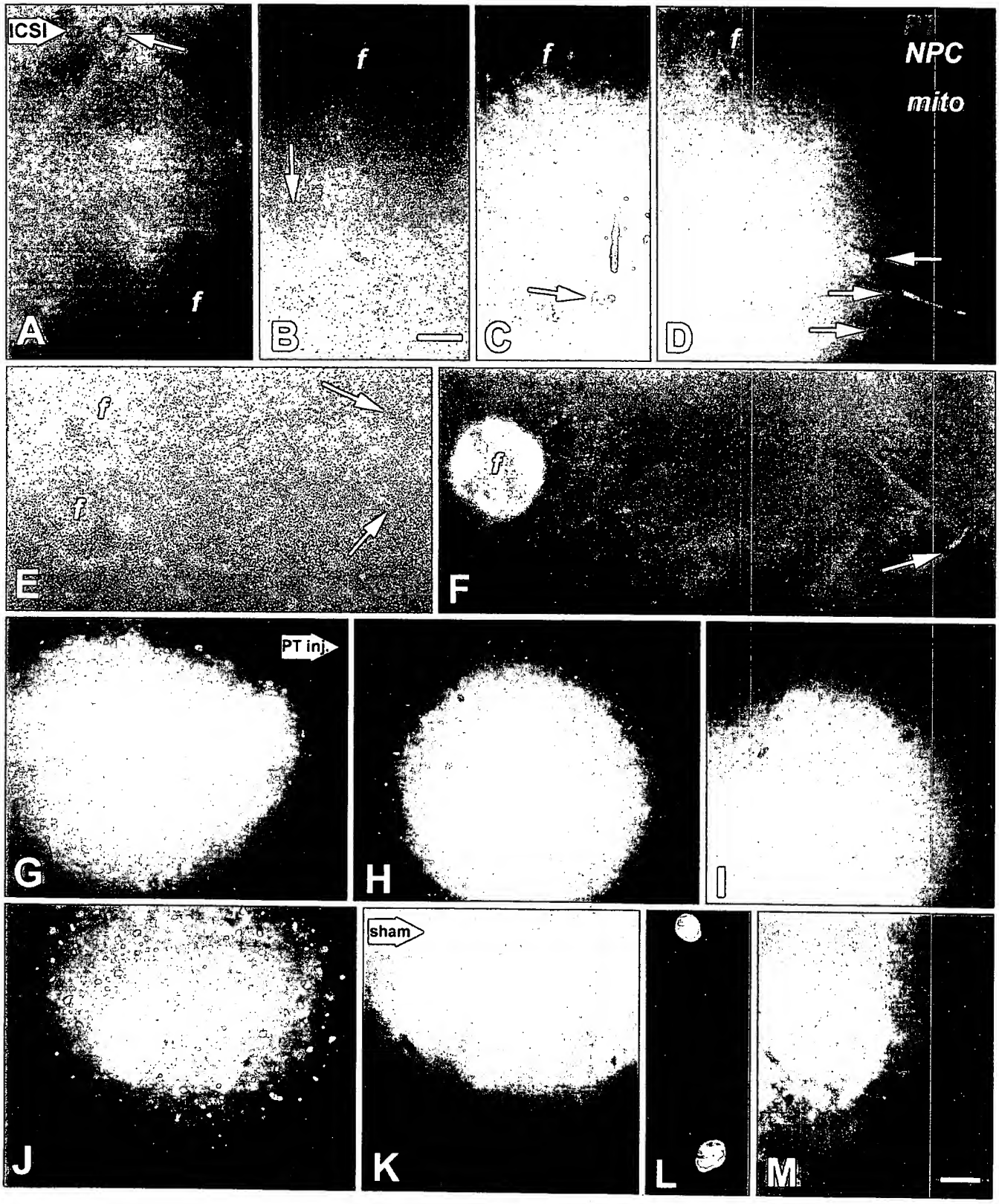


FIG 4

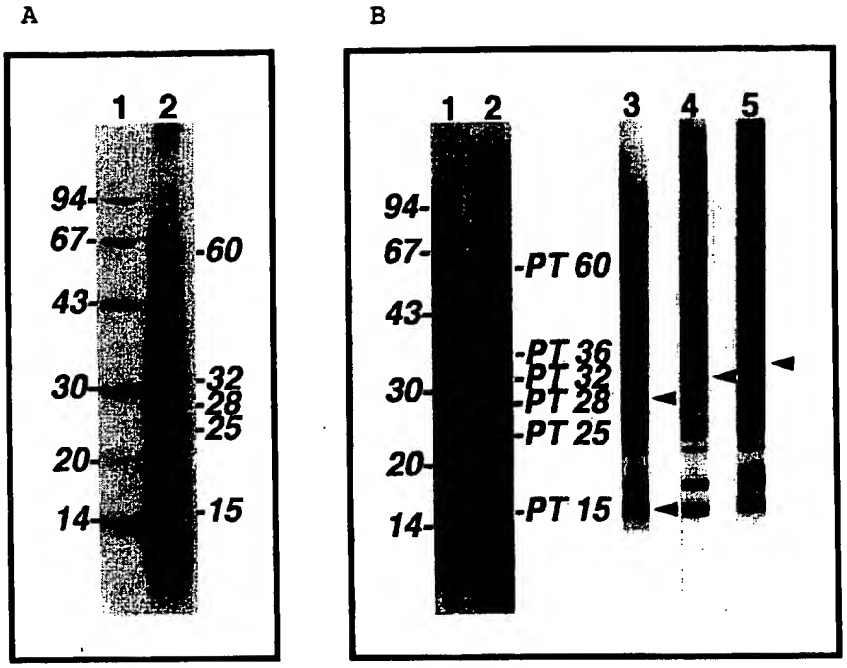

A
1 2
94
67
43
30
20
14
-60
-32
-28
-25
-15
B
1 2 3 4 5
94
67
43
30
20
14
-PT 60
-PT 36
-PT 32
-PT 28
-PT 25
-PT 15

FIG 5

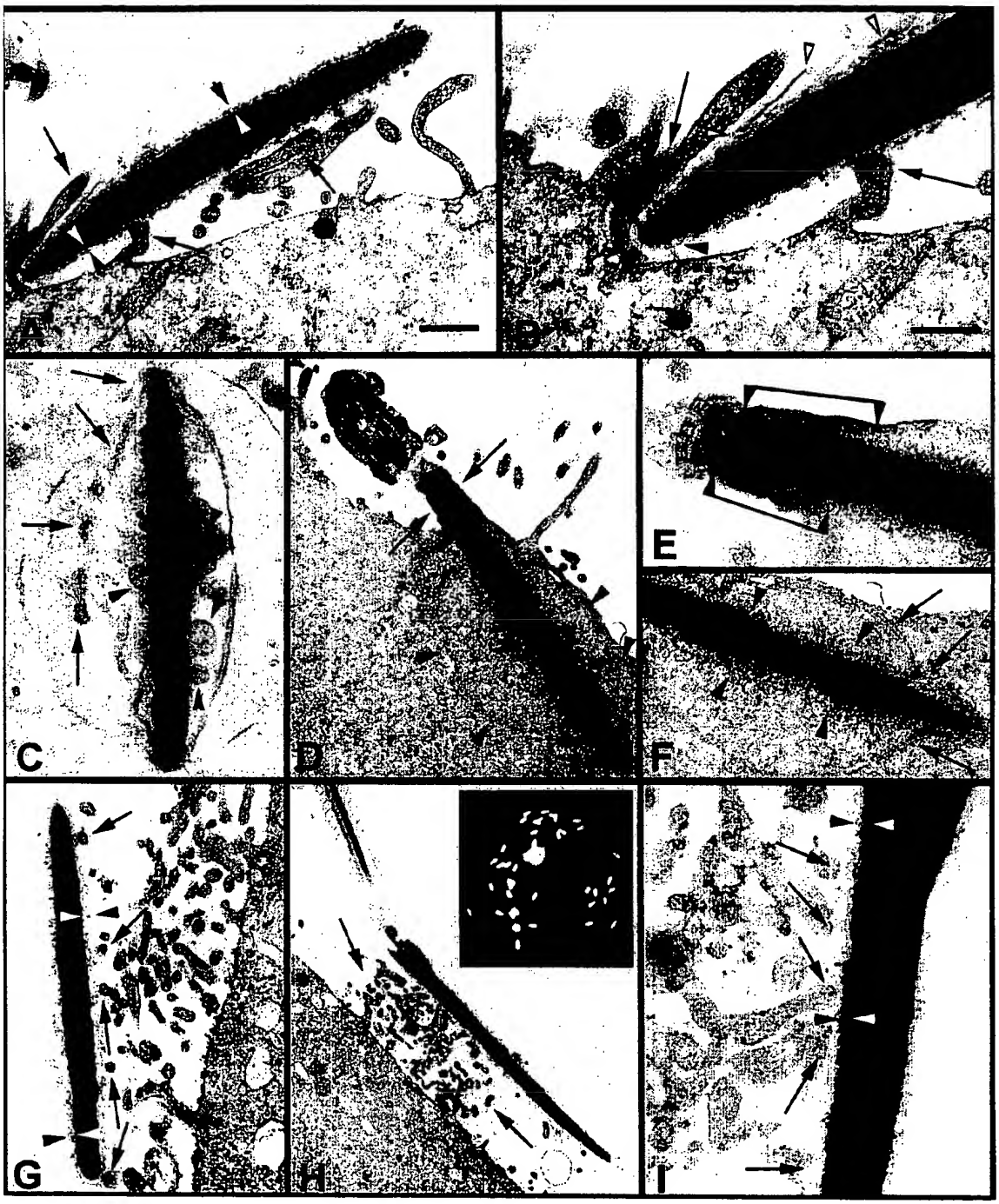


FIG 6

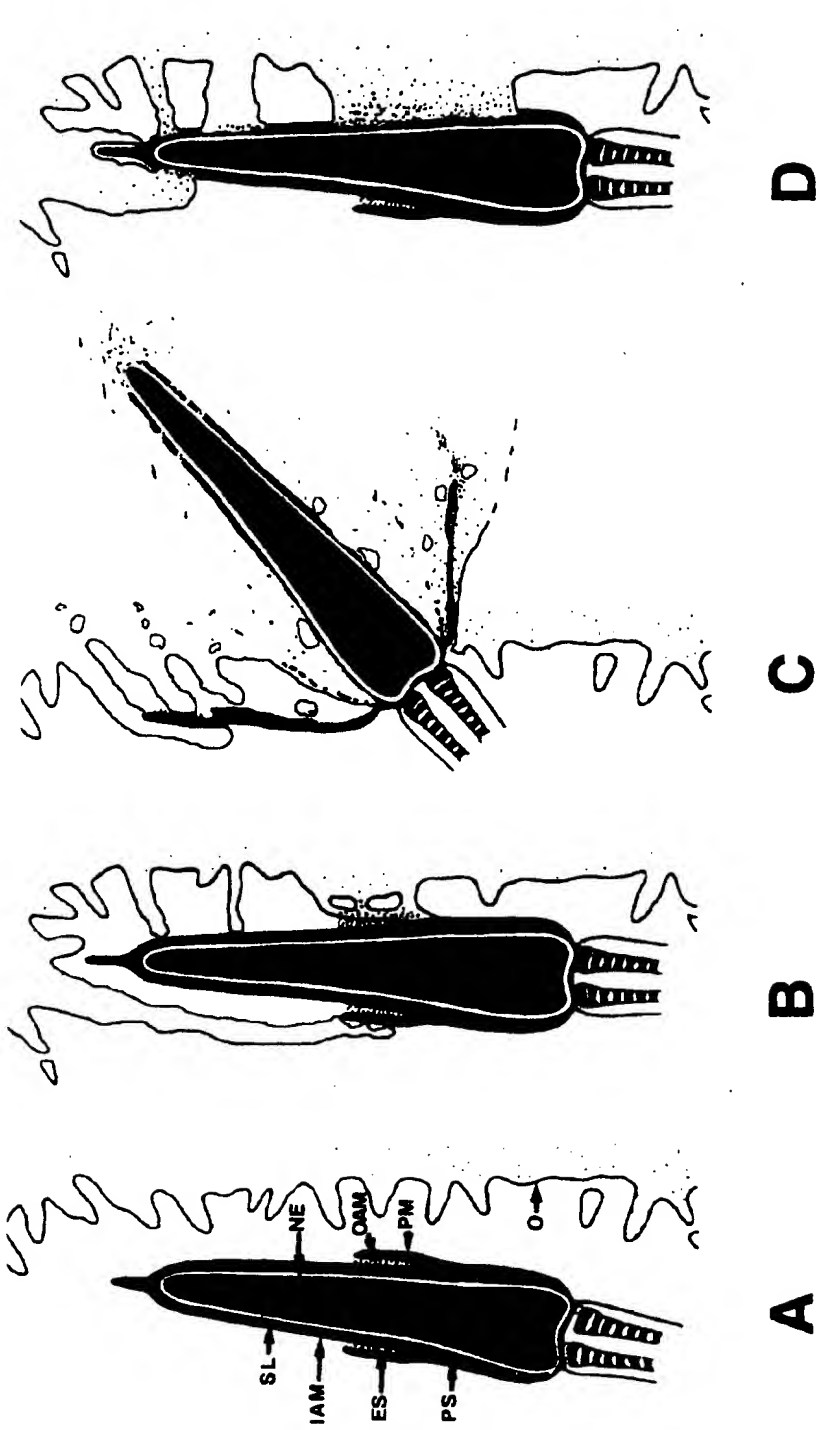
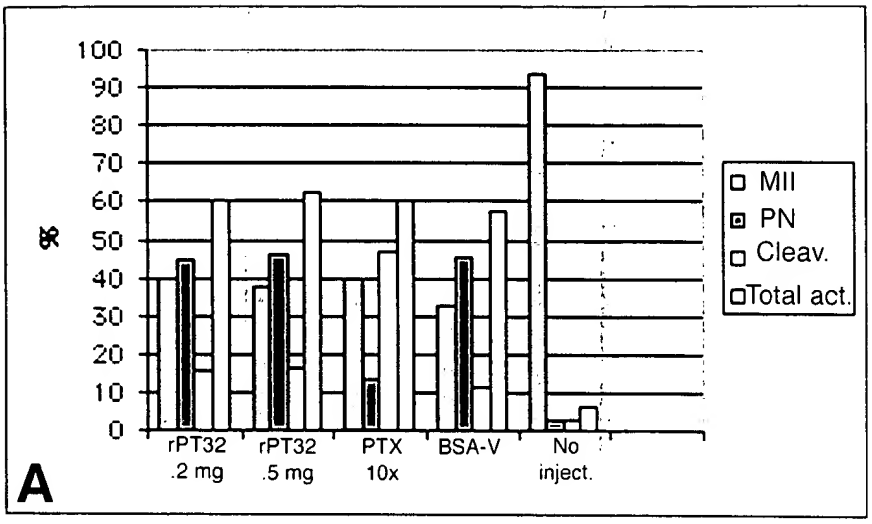


FIG 7


100
90
80
70
60
50
40
30
20
10
0
%
rPT32 .2 mg
rPT32 .5 mg
PTX 10x
BSA-V
No inject.
MII
PN
Cleav.
Total act.
A

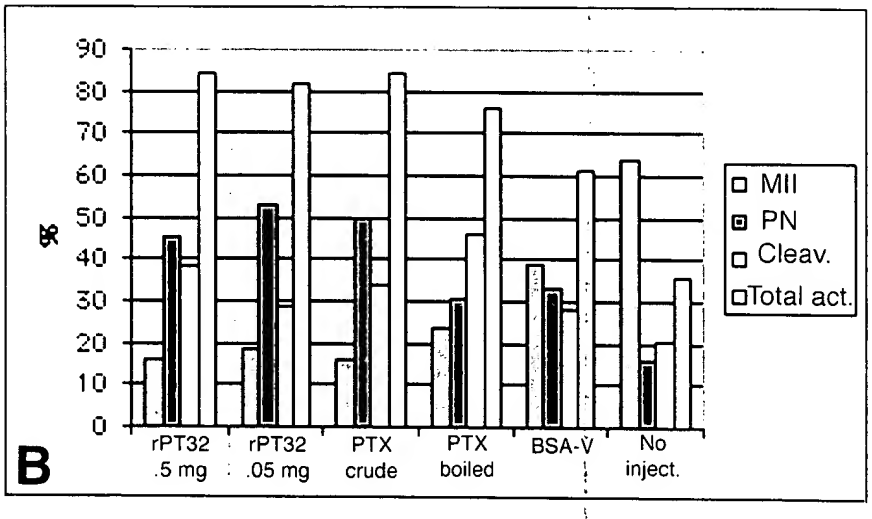

90
80
70
60
50
40
30
20
10
0
%
rPT32 .5 mg
rPT32 .05 mg
PTX crude
PTX boiled
BSA-V
No inject.
MII
PN
Cleav.
Total act.
B

FIG 8

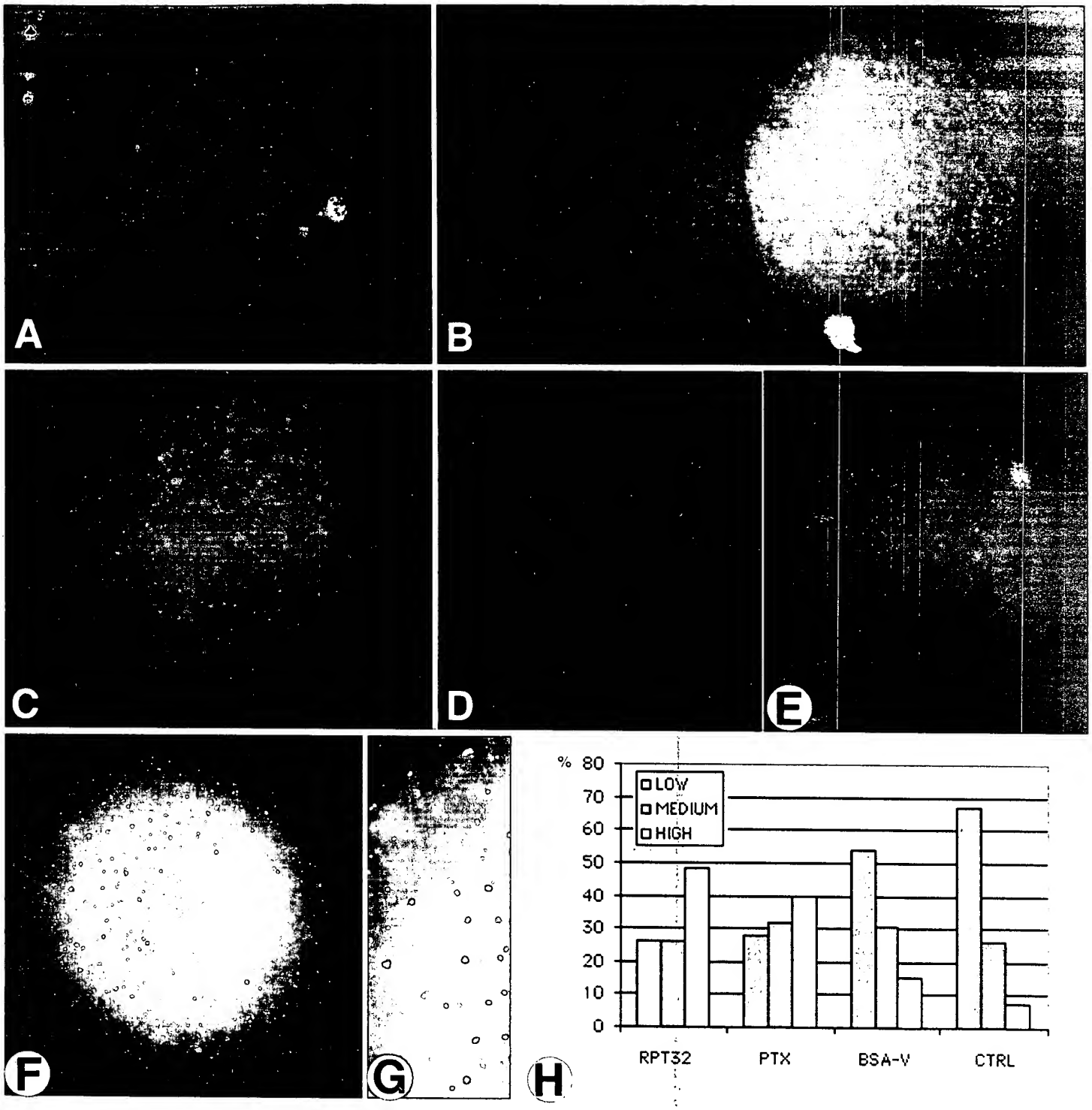

A
B
C
D
E
F
G
H
% 80
70
60
50
40
30
20
10
0
LOW
MEDIUM
HIGH
RPT32
PTX
BSA-V
CTRL

FIG 9

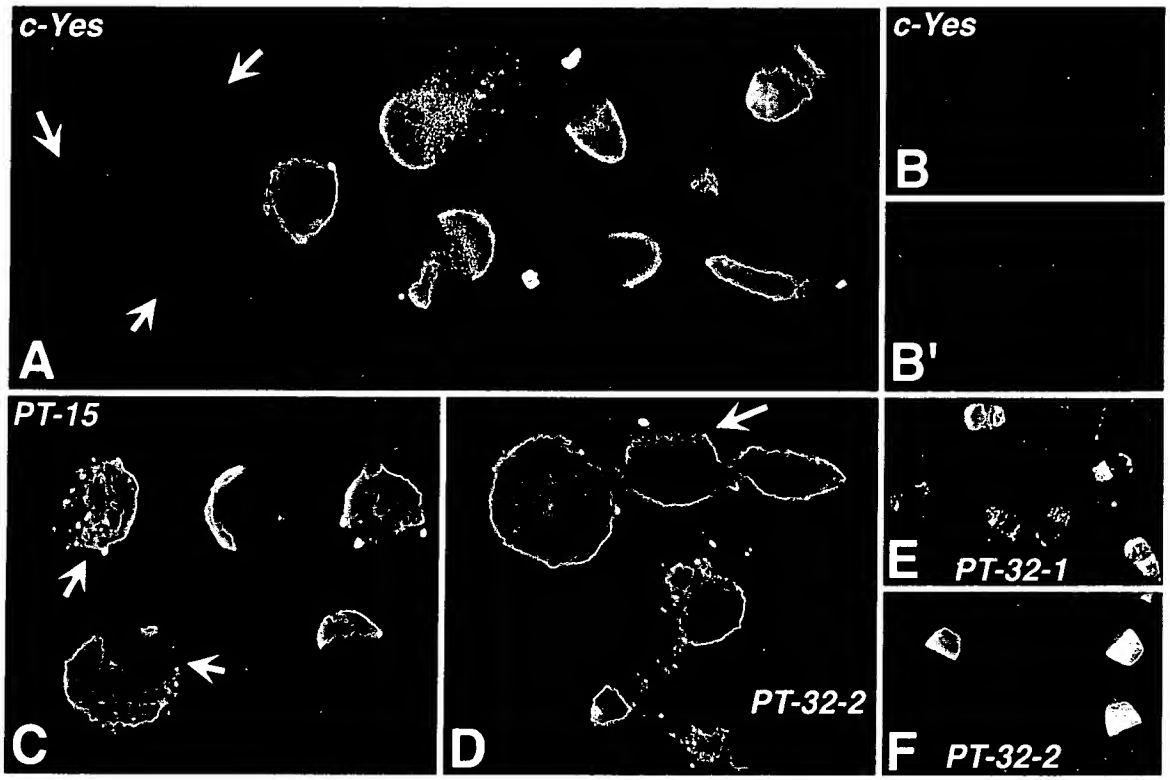


FIG 10

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             the EMBL outstation - the European Bioinformatics Institute.
             The original entry is available from http://www.expasy.ch/sprot
             and http://www.ebi.ac.uk/sprot
             ---------------------------------------------------------------------.
             [CATALYTIC ACTIVITY] ATP + A PROTEIN TYROSINE = ADP + PROTEIN
             TYROSINE PHOSPHATE.
             [SIMILARITY] CONTAINS 1 SH2 DOMAIN.
             [SIMILARITY] CONTAINS 1 SH3 DOMAIN.
             [SIMILARITY] TO OTHER PROTEIN-TYROSINE KINASES IN THE CATALYTIC
             DOMAIN. BELONGS TO THE SRC SUBFAMILY.
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     Site            396
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     Site            424
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ORIGIN
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       61 ggpsgmtpfg gassafsavp spypstltgg vtvfvalydy earttddlsf kkgerfqiin
      121 ntegdwwear siatgktgyi psnyvapads iqseewyfgk mgrkdaerll lnpgnqrgif
      181 lvresettkg ayslsirdwd evrgdnvkhy kirkldnggy yittraqfes lqklvkhyse
      241 hadglchklt tvcptvkpqt qglakdawei preslrlevk lgqgcfgevw mgtwngttkv
      301 aiktlkpgtm mpeaflqeaq imkklrhdkl vplyavvsee piyivtefmt kgslldflke
      361 gegkflklpq lvdmaaqiad gmayiermny ihrdlraani lvgdnlvcki sdfglarlie
      421 dneytarqga kfpikwtape aalygrftik sdvwsfgill telvtkgrvp ypgmvnrevl
      481 eqvergyrmp cpqgcpeslh elmklcwkkd pderptfeyi qsfledyfta tepqyqpgdn
      541 l
```

FIG 11